JOURNAL OF BACTERIOLOGY, Apr. 1999, p. 2358–2362
0021-9193/99/$04.00+0

Vol. 181, No. 8

Genetic Diversity in the Protective Antigen Gene of *Bacillus anthracis*

LANCE B. PRICE,[1] MARTIN HUGH-JONES,[2] PAUL J. JACKSON,[3] AND PAUL KEIM[1]*

Department of Biological Science, Northern Arizona University, Flagstaff, Arizona 86011-5640[1]; Department of Epidemiology and Community Health, School of Veterinary Medicine, Louisiana State University, Baton Rouge, Louisiana 70803[2]; and Environmental Molecular Biology Group, Los Alamos National Laboratory, Los Alamos, New Mexico 87545[3]

Received 2 December 1998/Accepted 29 January 1999

***Bacillus anthracis* is a gram-positive spore-forming bacterium that causes the disease anthrax. The anthrax toxin contains three components, including the protective antigen (PA), which binds to eucaryotic cell surface receptors and mediates the transport of toxins into the cell. In this study, the entire 2,294-nucleotide protective antigen gene (*pag*) was sequenced from 26 of the most diverse *B. anthracis* strains to identify potential variation in the toxin and to further our understanding of *B. anthracis* evolution. Five point mutations, three synonymous and two missense, were identified. These differences correspond to six different haploid types, which translate into three different amino acid sequences. The two amino acid changes were shown to be located in an area near a highly antigenic region critical to lethal factor binding. Nested primers were used to amplify and sequence this same region of *pag* from necropsy samples taken from victims of the 1979 Sverdlovsk incident. This investigation uncovered five different alleles among the strains present in the tissues, including two not seen in the 26-sample survey. One of these two alleles included a novel missense mutation, again located just adjacent to the highly antigenic region. Phylogenetic (cladistic) analysis of the *pag* corresponded with previous strain grouping based on chromosomal variation, suggesting that plasmid evolution in *B. anthracis* has occurred with little or no horizontal transfer between the different strains.**

Bacillus anthracis is the causative organism of the potentially fatal disease anthrax. Virulent forms of *B. anthracis* carry two large plasmids, pX01 (ca. 174 kb) and pX02 (ca. 95 kb). Virulence factors include toxin and capsule production, encoded on pX01 and pX02, respectively. The anthrax toxin is composed of three proteinaceous subunits: (i) lethal factor (LF), the toxin component thought to kill host cells by disrupting the mitogen-activated protein kinase pathway (2); (ii) edema factor (EF), an adenylyl cyclase that causes skin edema in the infected host (6); and (iii) protective antigen (PA), which binds to eucaryotic cell surface proteins, forms homoheptamers, and then binds to and internalizes EF and LF.

The structure and function of PA have been well described. The entire PA gene (*pag*) sequence has been published and is available in GenBank (accession no. M22589) (12). The three-dimensional structure has also been solved and is available in the NCBI Entrez 3D database (MMDB no. 6980) (10). Finally, antibody-binding experiments have been used to define regions of the PA protein critical to cell surface attachment as well as LF binding (8). Missing from the literature until now was a population study of *pag* from diverse strains of *B. anthracis* to define the natural variation in this important gene.

In past studies, plasmid-specific genetic variation in *B. anthracis* has been largely ignored. A recent population study, based on chromosomal markers, demonstrated that *B. anthracis* is one of the most monomorphic bacterial species known (5). This chromosomal amplified fragment length polymorphism study examined ca. 6.3% of the *B. anthracis* genome for length variations and ca. 0.36% for point mutations. However, due to ambiguities arising from the absence of one or both of the plasmids, plasmidal data were omitted from the final results. Studies of pX01 diversity and especially of *pag* are essential to understanding evolution of pathogenesis in *B. anthracis*. Likewise, comparative studies of plasmid-based versus chromosomal variation can provide insight into the frequency of horizontal plasmid transfer in natural *B. anthracis* populations.

In this study we have sequenced the entire *pag* gene from 26 of the most diverse strains of *B. anthracis* (5). These sequences were aligned and analyzed for point mutations then studied phylogenetically to determine if the *pag* data are consistent with chromosomal diversity groups. Additionally, we sequenced a 307-bp variable region of *pag* from 10 Sverdlovsk anthrax victim necropsy samples (4) in order to identify novel *pag* sequences.

MATERIALS AND METHODS

***B. anthracis* DNA.** Culture conditions, DNA isolation methods, and diversity groups are described in reference 5. Necropsy tissue DNA was isolated as described by Jackson et al. (4).

PCR amplification of DNA. Table 1 contains the sequences for all primers used for this project. These were designed from the published *pag* sequence (GenBank accession no. M22589) and synthesized by Gibco/BRL, Bethesda, Md. All primer positions cited throughout this report are based on this GenBank sequence. Two DNA fragments, together totaling 2,531 bp of sequence, were initially amplified to provide a *pag* sequencing template from the 26 *B. anthracis* strains. PA-1F and PA-1R were used to amplify a 1,191-bp fragment containing the 5′ portion of PA. This included 131 bp of upstream flanking sequence. PA-2F and PA-2R were used to amplify a 1,449-bp fragment containing the 3′ portion of PA. This included 106 bp of downstream flanking sequence. The two fragments contained 109 bp of overlapping sequence near the middle of the gene. Fifty-microliter PCR mixtures contained 1× PCR buffer (20 mM Tris [pH 8.4], 50 mM KCl; Gibco/BRL), 0.10 mM deoxynucleoside triphosphates, 4 mM $MgCl_2$, ~0.2 ng of template DNA per μl, 0.04 U of *Taq* DNA polymerase (Gibco/BRL) per μl, and 0.4 μM forward and reverse primers, adjusted to 50 μl with filtered (0.2-μm-pore-size filter) 17.8 mohm E-pure water. Reactions were heated to 94°C for 5 min and then subjected to 35 cycles, each consisting of 30 s at 94°C, 30 s at 62°C, and 1.5 min at 72°C. This was followed by heating to 72°C for 5 min to complete primer extension. PCR products were purified through

* Corresponding author. Mailing address: Department of Biological Sciences, Northern Arizona University, Flagstaff, AZ 86011-5640. Phone: (520) 523-1078. Fax: (520) 523-7500. E-mail: Paul.Keim@nau.edu.

TABLE 1. Primers used in this study

Primer	Type[a]	Sequence (5′→3′)	Position[b]	Amplicon size (bp)
PA-1F	Amp/Seq	ATA TTT ATA AAA GTT CTG TTT AAA AAG CC	5′-1673	1,191
PA-1R	Amp/Seq	TAA ATC CTG CAG ATA CAC TCC CAC	3′-2840	1,191
PA-2F	Amp/Seq	ATA AGT AAA AAT ACT TCT ACA AGT AGG ACA C	5′-2755	1,449
PA-2R	Amp/Seq	GAT TTA GAT TAC TGT TTA AAA CAT ACT CTC C	3′-4173	1,449
PA-3	Seq	TCA TGT AAC AAT GTG GGT AGA TGA C	5′-2145	NA[c]
PA-4	Seq	CTC TAT GAG CCT CCT TAA CTA CTG AC	3′-3717	NA
PA-5F	Amp	ATC CTA GTG ATC CAT TAG AAA CGA C	5′-3416	330
PA-5R	Amp	CTT CTC TAT GAG CCT CCT TAA CTA CTG	3′-3719	330
$PA\text{-}5F_{nest}$	Amp/Seq	AGT GAT CCA TTA GAA ACG AC	5′-3421	307
$PA\text{-}5R_{nest}$	Amp/Seq	TAA CTA CTG ACT CAT CCG C	3′-3709	307

[a] Amp, used for amplification; Seq, used for sequencing; Amp/Seq, used for both amplification and sequencing.
[b] All correspond to GenBank accession no. M22589 nucleotide positions.
[c] NA, not applicable.

Qiaquick purification minicolumns (Qiagen Inc., Valencia, Calif.) and then quantified on ethidium bromide-stained 1.25% agarose-Tris-acetate-EDTA gels. These purified fragments were then used in subsequent sequencing reactions. PCR amplification of necropsy sample DNA was performed as described by Jackson et al. (4), using primers PA-5F, PA-5R, $PA\text{-}5F_{nest}$, and $PA\text{-}5R_{nest}$ (Table 1).

DNA sequencing. PCR products were sequenced on an ABI model 377 fluorescence sequencer using a PRISM Ready Reaction BigDye terminator cycle sequencing kit (both from Perkin-Elmer/Applied Biosystems Inc., Foster City, Calif.). Sequences were aligned and analyzed with Sequence Navigator software (Perkin-Elmer/Applied Biosystems).

Cladistic analysis. Cladistic analysis was performed on the *pag* sequences by using maximum parsimony with PAUP 3.1.1 software (developed by David L. Swofford, Illinois Natural History Survey) and manual examinations of sequence polymorphisms.

Three-dimensional analysis. The PA structure has been solved and is available on the NCBI Entrez 3D database (MMDB no. 6980) (10). Amino acid residues shown to vary among strains were identified on the three-dimensional structure, and then physical distances from the putative LF binding region of PA domains 3 and 4 were estimated by using MAGE 4.5 software (developed by David Richardson, Biochemistry Department, Duke University, Durham, N.C.).

RESULTS

Sequence alignment of the entire PA gene from 26 strains representative of the five *B. anthracis* diversity groups (5) (Table 2) revealed five point mutations, three synonymous and two missense, shown in Table 3. All five mutations are transitions. Two of the synonymous mutations occur only once. However, the other differences are present with frequencies ranging from 3/26 to 20/26. The two missense mutations are located adjacent to a highly antigenic region crossing the junction between PA domains 3 and 4 shown to be critical to LF binding (Fig. 1) (8, 10). The different mutational combinations observed in this

TABLE 2. *B. anthracis* strains used in this study

Strain	Geographic origin or description	Diversity group[a]	PA genotype[b]	PA phenotype[c]
BA0052	Jamaica	Sterne-Ames	I	FPA
BA1087	Scotland	Sterne-Ames	I	FPA
J611	Indonesia	Sterne-Ames	I	FPA
BA1031	South Africa	Sterne-Ames	I	FPA
BA1043	South Africa	Sterne-Ames	I	FPA
28	Ohio	Sterne-Ames	II	FPA
MOZ-3	Mozambique	Southern Africa	III	FPA
BA1035	South Africa	Southern Africa	III	FPA
33	South Africa	Southern Africa	IV	FPA
A24	Slovakia	Southern Africa	V	FPV
K20	South Africa (Kruger)	Kruger	V	FPV
26/05/94	Zambia	Kruger	V	FPV
BA1033	South Africa	WNA	V	FPV
BA1017	Haiti	WNA	V	FPV
BA1015	Maryland	WNA	V	FPV
93-194C	Canada	WNA	V	FPV
93-195C-8	Canada	WNA	V	FPV
BA1040	Colorado	WNA	V	FPV
BA1007	Iowa	WNA	V	FPV
2/6	Turkey	WNA	V	FPV
Pak-2	Pakistan	WNA	V	FPV
STI-1	Russian vaccine strain	WNA	V	FPV
F-1	South Korea	Vollum	V	FPV
BA1024	Ireland	Vollum	VI	FSV
ASC-3	United Kingdom	Vollum	VI	FSV
BA1009	Pakistan	Vollum	VI	FSV

[a] Diversity designations are consistent with those described by Keim et al. (5).
[b] Described in Table 4.
[c] Designated by the single-letter designations of the three amino acids shown to vary in this study.

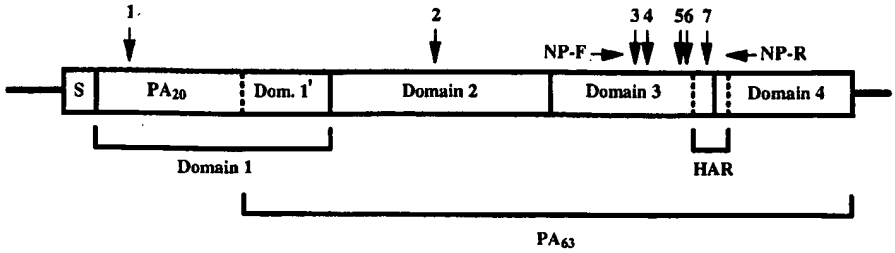

FIG. 1. Model of *pag* from *B. anthracis*. S, region of gene that codes for cleaved signal region; NP-F and NP-R, forward and reverse nested primers used to amplify variable regions from the Sverdlovsk tissue samples; black vertical arrows, missense mutations; grey vertical arrows, synonymous mutations; HAR, highly antigenic region important to LF binding (8, 10). Dom., domain.

study give rise to six PA genotypes and three PA phenotypes (Table 4).

Cladistic analysis of the 26 *pag* sequences was performed by the maximum parsimony method to produce a gene tree (Fig. 2A). The 26 strains grouped into four clades of 3, 3, 6, and 14 individuals. These groups were defined by three synapomorphic (informative) differences. In addition, we identified two apomorphic (uninformative) nucleotide differences (mutations 2 and 6) that separated two strains (28 and 33) from others in their clades. These mutations are identified on the respective branches but were not used to isolate these strains from their groups. The clades and topology identified by this tree were mostly congruent with those generated from chromosomal markers (Fig. 2B) (5). The only aberrations are the following. (i) Chromosomal data from strain A24 indicate that it is of the Southern Africa lineage (5), but the *pag* data place this strain with the Western North America (WNA) diversity group (one mutational step away); (ii) chromosomal data from strain F-1 indicate that it is of the Vollum lineage, but the *pag* data place this strain with the WNA diversity group (again, one mutational step away); and (iii) chromosomal markers indicate that the Kruger samples, although very similar, are genetically distinct from the WNA lineage. However, the *pag* gene tree did not resolve these two distinct groups. It should be noted that chromosomal markers indicate that Vollum and WNA are sister groups and, likewise, that Kruger and WNA are closely related. Only with strain A24 do the *pag* data suggest that strains from two distantly related groups (based on chromosomal markers) are closely related.

To determine the *pag* genotypes and phenotypes of the strain(s) involved in the Sverdlovsk incident, nested PCR primers (Table 1) were designed to amplify and sequence a 307-bp region of *pag*. This region spans the junction between PA domains 3 and 4 where much of the variation was observed. This analysis uncovered two additional transition mutations (3 and 7 in Table 3). One was synonymous, while the other was a novel missense mutation resulting in a phenylalanine↔leucine change. These changes resulted in two additional genotypes and one new phenotype (Table 4). The amino acid change was, again, immediately adjacent to the highly antigenic region of PA domains 3 and 4 (Fig. 1). Repetitive sequencing of these tissues uncovered multiple PA genotypes within some of the individual necropsy samples. Together, five different PA genotypes were observed in the Sverdlovsk samples, with some samples showing evidence of infection by multiple strains (Table 5). This finding is consistent with the results of Jackson et al. (4).

Figure 3 is an unrooted phylogenetic tree demonstrating the five mutational steps leading to the six PA genotypes and three PA phenotypes identified in this study. Additionally, the putative positions of the Sverdlovsk samples are shown. However, because the Sverdlovsk identifications were based on just the 307-bp region around the antigenic portion of PA domains 3 and 4, these placements are only tentative.

Three-dimensional analysis of all the amino acid changes observed in this study (mutations 3, 4, and 5 in Table 3) indicated that these changes are not only close sequentially but also very close in three-dimensional space to the antigenic region important for LF binding. Mutation 3 (Phe to Leu), is ca. 11.2 Å, mutation 4 (Pro to Ser) is ca. 20.3 Å, and mutation 5 (Ala to Val) is ca. 19.0 Å from the central portion of this

TABLE 3. Mutations identified in this study

Mutation	Nucleotide position[a]	Base change	Frequency	Amino acid change
1	1998	C⇔T	20/26	Synonymous
2	2883	G⇔A	1/26	Synonymous
3	3481	T⇔C	NA[b]	F⇔L
4	3496	C⇔T	3/26	P⇔S
5	3602	C⇔T	17/26	A⇔V
6	3606	T⇔C	1/26	Synonymous
7	3672	A⇔G	NA	Synonymous

[a] Nucleotide positions are based on the 4,235-bp pX01 sequence from Sterne strain, accession no. M22589, containing *pag* in its entirety.
[b] NA, not applicable (mutation was observed only in the Sverdlovsk samples).

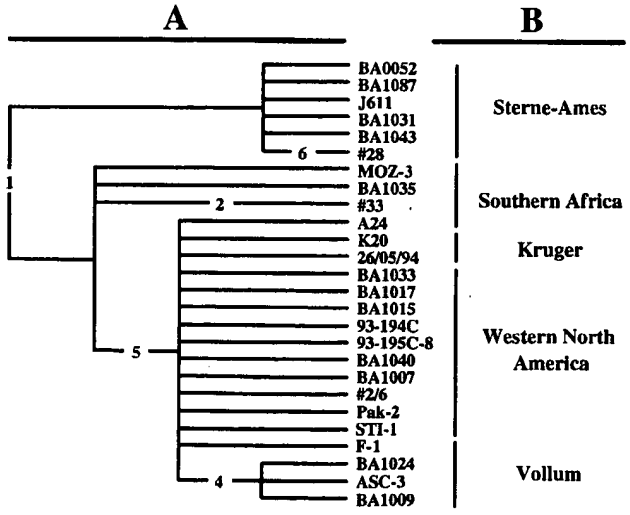

FIG. 2. Cladistic analysis of the 26 diverse strains. (A) Unrooted, maximum parsimony gene tree based on *pag* data developed in this study; (B) strain diversity groups based on chromosomal AFLP data described by Keim et al. (5). Branch mutations are numbered as described for Table 3.

TABLE 4. PA genotypes and phenotypes identified in this study[a]

PA genotype	PA phenotype[b]	Genotypic frequency	Mutation[c]						
			1	2	3	4	5	6	7
I	FPA	5/26	C	G	T(F)	C(P)	C(A)	T	A
II	FPA	1/26	C	G	T(F)	C(P)	C(A)	C	A
III	FPA	2/26	T	G	T(F)	C(P)	C(A)	T	A
IV	FPA	1/26	T	A	T(F)	C(P)	C(A)	T	A
V	FPV	14/26	T	G	T(F)	C(P)	T(V)	T	A
VI	FSV	3/26	T	G	T(F)	T(S)	T(V)	T	A
VII_{Svd}	LPA	NA	—	—	C(L)	C(P)	C(A)	T	A
$VIII_{Svd}$	FPA	NA	—	—	T(F)	C(P)	C(A)	T	G

[a] NA, not applicable (mutation was seen only in the Sverdlovsk samples); —, the region was not analyzed for the Sverdlovsk sample.
[b] Designated by the single-letter designation of the three amino acids shown to vary.
[c] Described in Table 3.

region. These spatial distances were estimated solely on peptide backbone-to-peptide backbone relationships. However, when the three-dimensional spaces occupied by the side chains of the amino acids were considered, changes were found to affect residues as close as 6.9 Å from the central amino acids of this critical antigenic region.

DISCUSSION

The protective antigen protein is central to the virulence associated with anthrax toxin. Elucidation of PA variation and its encoding gene could lead to a better understanding of *B. anthracis* virulence and evolution. Until now, *pag* had been sequenced in its entirety only from a single *B. anthracis* strain (12). In this study, a detailed analysis of the entire *pag* sequence (2,294 bp) from 26 diverse *B. anthracis* strains revealed only five point mutations, corroborating the high degree of genetic monomorphism found by Keim et al. (5).

Among these mutations, there is a disproportionate number of missense (two) to synonymous (three) changes. A common ratio of missense to synonymous mutations is approximately 1:5; here we see a ratio more than threefold greater (7). These missense mutations are located near a highly antigenic region, critical to LF binding. In monoclonal antibody studies, Little et al. demonstrated that by blocking an epitope between amino acids Ile-581 and Asn-601 (Fig. 1), they could effectively block LF binding to PA (8). Three-dimensional analysis indicated that the missense mutations identified in our study are very close in three-dimensional space to this antigenic region. While none of the three missense mutations were dramatic, such as a change from an extremely hydrophobic to a hydrophilic amino acid, the proline-to-serine change has the potential to make important three-dimensional alterations, since proline isomerization is known to play a critical role in protein folding. Because of their close proximity, these amino acid changes have the potential to effect LF binding, either directly or indirectly, within an infected host. The grouping of these missense mutations near this antigenic region and the disproportionate number of missense to synonymous mutations suggests adaptive variation. One of the two new mutations identified in the Sverdlovsk victims' tissues was found to be a novel missense mutation located, sequentially and three dimensionally, near the highly antigenic region of the junction between PA domains 3 and 4. When these mutations are included with those identified in the 26-sample survey, the ratio of missense to synonymous mutation is increased to 3.8:5.

The amplification and sequencing of the 307-bp *pag* fragment from the Sverdlovsk tissue samples suggested that at least five different strains of *B. anthracis* were present in the samples and that some of the individual victims had been infected with multiple strains. These data corroborate earlier work with the *vrrA* locus that suggested that multiple strains of anthrax had been released during the 1979 incident (1, 4). Besides the Russian vaccine strain STI-1, included in this study, these tissue samples are a rare glimpse at the different strains of *B. anthracis* that are thought to be endemic in the vast region of the former Soviet Union. The fact that two previously unobserved mutations were found in the Sverdlovsk samples stresses the importance of collecting and analyzing *B. anthracis*

TABLE 5. Tissue samples from Sverdlovsk victims analyzed in this study[a]

Sample	Tissue	PA genotype(s)	PA phenotype(s)
7.RA93.15.15	Spleen	V	FPV
40.RA93.40.5	Spleen	VI, VII_{Svd}	FSV, LPA
27.RA93.30.3	Spleen	V	FPV
37.RA93.35.4	Vaccination site	I,[b] V	FPA, FPV
37.RA93.35.6	Lung	$VIII_{Svd}$	FPA
3.RA93.1.1	Meninges	$VIII_{Svd}$	FPA
25.RA93.031	Meninges	V	FPV
1.RA93.42.1	Meninges	V	FPV
33.RA93.20.5	Meninges	V	FPV
21.RA93.38.4	Lymph node	V	FPV

[a] Determination of PA genotypes and phenotypes was based solely on the 307-bp region connecting PA domains 3 and 4. Multiple strains were identified in some tissues.
[b] Due to the limited region analyzed, this strain may be type I, II, or IV but was grouped with I for simplicity.

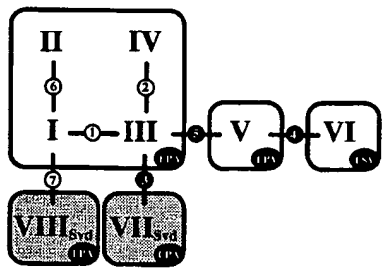

FIG. 3. Unrooted phylogenetic tree of PA genotypes. Open boxes show the three PA phenotypes identified; shaded boxes show the possible positions of the Sverdlovsk genotypes, VII_{Svd} and $VIII_{Svd}$. Synonymous mutations are shown in open circles, and missense mutations are shown in closed circles. Each mutation is described in Table 3 and the phenotypes are described in Table 4.

strains from areas where anthrax is endemic but largely uncharacterized by molecular genetic analysis.

Independent cladistic analysis of pX01 by using the *pag* sequence has enabled us to estimate the likelihood of horizontal transfer of this plasmid between different *B. anthracis* strains in natural populations. Although horizontal transfer in *Bacillus* spp. is possible under laboratory conditions, the similarity of the cladistic grouping from the *pag* data to that of the chromosomal markers suggests that the differences in *pag* arose from evolution within particular strain lineages and were not a result of horizontal pX01 transfer. The single possible exception is associated with the A24 sample, which chromosomally is related to the Southern Africa strains, while the *pag* data for this strain are consistent with Kruger-WNA. This is either a result of convergent evolution or evidence of horizontal pX01 transfer. Further, it should be noted that the data presented in this report do not rule out the potential for horizontal transfer of plasmid pX01 between closely related strains within an infected host.

The unrooted phylogenetic tree (Fig. 3) is a useful tool for demonstrating the relationships between the different PA genotypes. However, it is not meant to infer an evolution toward a particular form of PA. Although distant homologues from other gram-positive bacteria are cited (3, 9, 11), none of these is close enough to root a *B. anthracis* PA phylogenetic tree. Without an ancestral PA sequence, one is unable to determine which PA phenotypes are ancestral and which are derived.

ACKNOWLEDGMENTS

We thank James M. Schupp, Kimothy L. Smith, Debra M. Adair, and Karen K. Hill for technical support.

REFERENCES

1. **Andersen, G. L., J. M. Simchock, and K. H. Wilson.** 1996. Identification of a region of genetic variability among *Bacillus anthracis* strains and related species. J. Bacteriol. **178**:377–384.
2. **Duesbery, N. S., C. P. Webb, S. H. Leppla, V. M. Gordon, K. R. Klimpel, T. D. Copeland, N. G. Ahn, M. K. Oskarsson, K. Fukasawa, K. D. Paull, and G. F. Vande Woude.** 1998. Proteolytic inactivation of MAP-kinase-kinase by anthrax lethal factor. Science **280**:734–737.
3. **Gibert, M., S. Perelle, G. Daube, and M. R. Popoff.** 1997. *Clostridium spiroforme* toxin genes are related to *C. perfringens* iota toxin genes but have a different genomic localization. Syst. Appl. Microbiol. **20**:337–347.
4. **Jackson, P. J., M. E. Hugh-Jones, D. M. Adair, G. Green, K. K. Hill, C. R. Kuske, L. M. Grinberg, F. A. Abramova, and P. Keim.** 1998. PCR analysis of tissue samples from the 1979 Sverdlovsk anthrax victims: the presence of multiple *Bacillus anthracis* strains in different victims. Proc. Natl. Acad. Sci. USA **95**:1224–1229.
5. **Keim, P., A. Kalif, J. Schupp, K. Hill, S. E. Travis, K. Richmond, D. M. Adair, M. Hugh-Jones, C. R. Kuske, and P. Jackson.** 1997. Molecular evolution and diversity in *Bacillus anthracis* as detected by amplified fragment length polymorphism markers. J. Bacteriol. **179**:818–824.
6. **Leppla, S. H.** 1982. Anthrax toxin edema factor: a bacterial adenylate cyclase that increases cyclic AMP concentrations of eukaryotic cells. Proc. Natl. Acad. Sci. USA **79**:3162–3166.
7. **Li, W., and D. Graur.** 1991. Fundamentals of molecular evolution. Sinauer Associates, Inc., Sunderland, Mass.
8. **Little, S. F., J. M. Novak, J. R. Lowe, S. H. Leppla, Y. Singh, K. R. Klimpel, B. C. Ligerding, and A. M. Friedlander.** 1996. Characterization of lethal factor binding and cell receptor binding domains of protective antigen of *Bacillus anthracis* using monoclonal antibodies. Microbiology **142**:707–715.
9. **Perelle, S., M. Gibert, P. Boquet, and M. R. Popoff.** 1993. Characterization of *Clostridium perfringens* iota-toxin genes and expression in *Escherichia coli*. Infect. Immun. **61**:5147–5156.
10. **Petosa, C., R. J. Collier, K. R. Klimpel, S. H. Leppla, and R. C. Liddington.** 1997. Crystal structure of the anthrax toxin protective antigen. Nature **385**: 833–838.
11. **Selvapandiyan, A.** 1998. Direct submission. Genbank accession no. Y17158.
12. **Welkos, S. L., J. R. Lowe, F. Eden-McCutchan, M. Vodkin, S. H. Leppla, and J. J. Schmidt.** 1988. Sequence and analysis of the DNA encoding protective antigen of *Bacillus anthracis*. Gene **69**:287–300.

A cationic lipid-formulated plasmid DNA vaccine confers sustained antibody-mediated protection against aerosolized anthrax spores

G. Hermanson*†, V. Whitlow*, S. Parker*‡, K. Tonsky*, D. Rusalov*, M. Ferrari*, P. Lalor*, M. Komai*, R. Mere*, M. Bell§, K. Brenneman§, A. Mateczun¶, T. Evans*, D. Kaslow*, D. Galloway§¶, and P. Hobart*

*Vical Inc., San Diego, CA 92121; §Department of Microbiology, Ohio State University, Columbus, OH 43210; and ¶Naval Medical Research Center, Silver Spring, MD 20910

Communicated by C. Thomas Caskey, Cogene Biotech Ventures, Houston, TX, August 4, 2004 (received for review March 2, 2004)

DNA vaccines provide an attractive technology platform against bioterrorism agents due to their safety record in humans and ease of construction, testing, and manufacture. We have designed monovalent and bivalent anthrax plasmid DNA (pDNA) vaccines encoding genetically detoxified protective antigen (PA) and lethal factor (LF) proteins and tested their immunogenicity and ability to protect rabbits from an aerosolized inhalation spore challenge. Immune responses after two or three injections of cationic lipid-formulated PA, PA plus LF, or LF pDNAs were at least equivalent to two doses of anthrax vaccine adsorbed (AVA). High titers of anti-PA, anti-LF, and neutralizing antibody to lethal toxin (Letx) were achieved in all rabbits. Eight or nine animals in each group were challenged with 100× LD_{50} of aerosolized anthrax spores 5 or 9 weeks after vaccination. An additional 10 animals vaccinated with PA pDNA were challenged >7 months postvaccination. All animals receiving PA or PA plus LF pDNA vaccines were protected. In addition, 5 of 9 animals receiving LF pDNA survived, and the time to death was significantly delayed in the others. Groups receiving three immunizations with PA or PA plus LF pDNA showed no increase in anti-PA, anti-LF, or Letx neutralizing antibody titers postchallenge, suggesting little or no spore germination. In contrast, titer increases were seen in AVA animals, and in surviving animals vaccinated with LF pDNA alone. Preclinical evaluation of this cationic lipid-formulated bivalent PA and LF vaccine is complete, and the vaccine has received U.S. Food and Drug Administration Investigational New Drug allowance.

The deliberate exposure of the civilian population of the United States to *Bacillus anthracis* spores in late 2001 revealed several gaps in the public health system's preparedness against bioterrorism. Because vaccines have proven to be one of the most successful public health measures against naturally acquired infectious diseases, it is no surprise that a top biodefense priority is to develop vaccines against weaponized microorganisms. The performance requirements of biodefense vaccines, however, are somewhat different from those for conventional vaccines. Some key features of biodefense vaccines to be developed for civilian use include (*i*) the rapidity by which a protective immune response can be elicited; (*ii*) the degree to which the vaccine, when administered postexposure, improves the clinical course of an exposed person; (*iii*) a very high benefit-to-risk ratio of vaccination in all segments of the population; (*iv*) the speed and ease of manufacture, distribution, and administration; and (*v*) the inherent stability of the formulated, final filled product to allow for long-term stockpiling. None of the currently licensed vaccines against select bioterrorism agents meet all of these performance requirements, including the currently licensed anthrax vaccine: "The current anthrax vaccine is difficult to standardize, is incompletely characterized, and is relatively reactogenic (probably even more so because it is administered subcutaneously), and the dose schedule is long and challenging. An anthrax vaccine free of these drawbacks is needed, and such improvements are feasible" (1).

Providing evidence of a biodefense vaccine's effectiveness by classic clinical efficacy trials is likely to be neither feasible nor ethical so these vaccines must be evaluated by the regulatory agencies in a unique way. The "Animal Rule" (www.fda.gov/cber/summaries/120600bio19KG.ppt), in which preferably two animal species are used to demonstrate the biological activity of the product, may establish effectiveness of these vaccines. For anthrax, the aerosolized spore inhalation challenge of vaccinated rabbits will likely be one of the animal models used to prove effectiveness. The studies reported here demonstrate the effectiveness of both monovalent and bivalent, cationic lipid-formulated gene-based plasmid DNA (pDNA) vaccines, using this rabbit model of anthrax.

The virulence of *B. anthracis* in rabbits, non-human primates, and humans is primarily the result of a multicomponent toxin secreted by the organism (2). The toxin consists of three separate gene products, designated protective antigen (PA), lethal factor (LF), and edema factor (EF), that are encoded on a 184-kb plasmid designated pXO1 (3). PA83 [735 aa, molecular weight (MW) 82,684] is a single-chain protein that binds to a mammalian ubiquitous cell surface receptor (4). Cleavage by furin (or a furin-like enzyme) results in a 63-kDa receptor-bound product (5, 6) that multimerizes and can bind both the 90-kDa LF protein and the 89-kDa EF protein, which are subsequently endocytosed as a two-component complex (7). LF (776 aa, MW 90,237) is a zinc metalloprotease that, once internalized, cleaves several isoforms of mitogen-activated protein kinase kinases (Mek1, Mek2, and MKK3) and thereby disrupts signal transduction events within the cell (8). The LF protein, which when complexed with PA is referred to as Letx, is considered responsible for the rapid lethality of anthrax spore inhalation infection (2, 9) due to extensive tissue hypoxia accompanied by pleural edema (9).

Recently, a pDNA vaccine encoding a truncated PA antigen (PA63) was shown to protect against Letx challenge in mice (10), and a plasmid encoding full-length PA protected 9 of 10 rabbits from s.c. spore challenge (11). Additionally, Price *et al.* (12) demonstrated that a pDNA encoding a fragment of the LF gene can contribute to, or provide protection against, Letx challenge, 5an observation subsequently extended in a small inhalation challenge rabbit study showing that a pDNA prime followed by

Freely available online through the PNAS open access option.

Abbreviations: pDNA, plasmid DNA; LF, lethal factor; PA, protective antigen; AVA, anthrax vaccine adsorbed; Letx, anthrax lethal toxin; DMRIE, (±)-*N*-(2-hydroxyethyl)-*N*,*N*-dimethyl-2,3-bis(tetradecyloxy)-1-propanaminium bromide; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; GMT, geometric mean titer; hTPA, human tissue plasminogen activator.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AY428556–AY428558).

†To whom correspondence should be addressed. E-mail: ghermanson@vical.com.

‡Present address: Biogenldec, San Diego, CA 92191.

a recombinant protein boost provided protection not seen with pDNA alone (13).

Given these considerations, we developed and tested a series of anthrax pDNA vaccine constructs. Three PA and LF pDNAs formulated with cationic lipid warranted further investigation in a lethal spore challenge study in rabbits. This work demonstrated that formulated pDNA vaccines induce complete, long-term protection, and that an LF pDNA administered alone provides partial protection in a model in which death results a few days after inhalation exposure (14). In contrast to the anthrax vaccine adsorbed (AVA) and LF pDNA alone groups, the formulated PA or bivalent PA plus LF pDNA vaccines generated a protective response at peak titer that seemed to block germination of the spores or sufficient bacterial replication to cause a postchallenge increase in anti-PA-, anti-LF-, or Letx-neutralizing titers.

Materials and Methods

Plasmids. ***PA83Δfurin.*** The nucleotide sequence encoding *B. anthracis* PA83 protein (GenBank accession no. AF306782, nt 49–2343) was codon-optimized with the backtranslation tool at www.syntheticgenes.com by using the *Homo sapiens* codon frequency table at www.kazusa.or.jp/codon. The PA construct (GenBank accession no. AY428556) was chemically synthesized (Retrogen, San Diego) to include an amino terminal human tissue plasminogen activator (hTPA) leader peptide (replacing the *Bacillus* leader peptide) fused to a PA83 sequence (amino acids 30–764) with the furin cleavage site deleted (SRKKRS, amino acids 192–197) (6, 15). This construct, designated PA83Δ furin, was cloned into the mammalian expression vector VR1012 for these studies (16).

LF[I] and LF[I–III]. The LF coding sequences used in this study were derived from the *B. anthracis* LF93 protein sequence (GenBank accession no. M30210, nt 784–3114), codon-optimized, and chemically synthesized as above to include the hTPA leader peptide. The LF domain I–III (17) was PCR amplified from this clone by using a forward (5′-GAGCTTGATATCGCCACCATGGATGC-3′) and reverse (5′-GAACCTGGATCCCTACACCACCTTGGCGTCGATG-3′) primer pair to amplify the 1,740-bp fragment (GenBank accession no. AY428557) encoding the hTPA leader peptide fused to LF amino acids 34–583. The LF domain [I] was also derived from the LF93 plasmid by PCR amplification using forward (5′-GAGCTTGATATCGCCACCATGGATGC-3′) and reverse (5′-CCATACGGATCCTCACTGGTCTTTCAGTTCCTCCA-3′) primer pairs to amplify an 876-bp fragment (GenBank accession no. AY428558) encoding an hTPA leader peptide fused to LF amino acids 34–295. Both LF genes were cloned into the VR1012 vector.

Plasmid DNA Preparation. Plasmid DNA was prepared from overnight cultures of transformed XL-2 Blue bacteria (Stratagene) in Terrific Broth (Invitrogen) plus 50 μg/ml kanamycin sulfate (Sigma) and processed by using Endo-free Giga kits (Qiagen, Valencia, CA).

Vaxfectin and DMRIE/DOPE Formulations. One milliliter of sterile water for irrigation (SWFI) was added to a vial containing a dried film of 3.75 μmol each of a 1:1 mixture of cationic lipid and colipid and vortex mixed for 5 min. Vaxfectin consists of GAP-DMORIE [(±)-*N*-(3-aminopropyl)-*N*,*N*-dimethyl-2,3-bis(*cis*-9-tetradecenyloxy)-1-propanaminium bromide)] plus DPyPE (1,2-diphytanoyl-*sn*-glycero-3-phosphoethanolamine). DMRIE/DOPE consists of DMRIE [(±)-*N*-(2-hydroxyethyl)-*N*,*N*-dimethyl-2,3-bis(tetradecyloxy)-1-propanaminium bromide] plus DOPE (1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine). The liposome suspension was diluted to 1.5 mM with SWFI and added to an equal volume of pDNA (2 mg/ml in 2× PBS) and vortex mixed briefly. The final molar ratio of all formulations was 4:1, DNA/cationic lipid.

Rabbit Intramuscular Vaccination. Two- to five-kilogram female New Zealand White rabbits were injected bilaterally in the quadriceps muscle with 1 ml (1 mg/ml) of pDNA formulated with Vaxfectin or DMRIE/DOPE (0.5 ml per leg). Rabbits vaccinated with PA, LF, or vector received 1 mg of that pDNA whereas rabbits coinjected with PA plus LF pDNAs received a mixture of 0.5 mg of each plasmid. Groups of rabbits receiving three doses were injected on days 0, 28, and 56; rabbits receiving only two doses were injected on study days 0 and 28. Rabbits immunized with AVA (a gift from BioPort Corporation, Lansing, MI, to D.G.) were injected unilaterally with 50 μl of AVA diluted to 0.5 ml in PBS on days 28 and 56. Prebleeds and biweekly postvaccination bleeds were taken for all groups for analysis of serum antibodies.

Anti-PA and Anti-LF Binding Antibody Titers. Anti-PA and anti-LF IgG antibody titers were determined by end-point dilution ELISA (18). Briefly, 96-well plates were coated with 1 μg/ml PA or LF protein (List Biological Laboratories, Campbell, CA). After blocking overnight, serum samples were serially diluted and incubated for 16 h at 4°C. Bound antibodies were detected with alkaline phosphatase-conjugated anti-rabbit IgG (1:4,000, H+L, Jackson ImmunoResearch) before adding the PNPP substrate (Sigma) and measuring absorbance at 450 nm (A_{450}). The average of duplicate wells was used to determine the end-point titer, defined as the reciprocal of the highest dilution giving a reading ≥3 times the control wells without serum.

Letx Neutralization Titers. Letx neutralizing antibody titers were measured by the J774A.1 mouse macrophage cytotoxicity assay (12). Two-fold dilutions of serum in DMEM (DMEM/10% FBS/1% sodium pyruvate) were incubated with an equal volume of Letx (500 ng/ml PA plus 400 ng/ml LF in DMEM) for 1 h at room temperature. The Letx plus serum mixture was added to wells containing 3×10^4 J774A.1 cells (plated 1 day before) for 4 h at 37°C, then replaced with 0.1 ml DMEM and 0.05 ml XTT reagent (Cell Proliferation Kit II, Roche Molecular Biochemicals). Cell viability was determined by measuring the A_{450} after 16 h. The A_{450} of 100% viable cells was calculated from the average of four wells receiving no Letx. The average of duplicate samples was used to calculate titers, defined as the reciprocal of the highest dilution of serum that gives an $A_{450} \geq 90\%$ of the value of wells receiving no Letx.

Anthrax Aerosolized Spore Challenge. In a blinded study at Battelle Memorial Institute (West Jefferson, OH) under BSL-3 conditions, selected rabbits (see Table 2 legend) were randomly divided into four groups and challenged with a target dose of 100× the LD_{50} of aerosolized *B. anthracis* spores, Ames strain, on successive days. Each group contained pDNA-vaccinated, AVA-vaccinated, vector controls, and naive rabbits. Samples of the spore-containing aerosol were collected to calculate the dose of exposure (1 Ames LD_{50} equals 105,000 colony-forming units) (13). After challenge, rabbits were observed for 21 days for signs of *B. anthracis* infection, and serum from surviving animals was collected 7 and 21 days postchallenge.

Statistical Analysis. Statistical analysis was by paired Student's *t* test, one-way ANOVA with pairwise comparisons, and two-way ANOVA with pairwise comparisons. $P < 0.05$ was considered significant. Analyses were performed with STATISTICAL ANALYSIS SYSTEM 8.02 (SAS Institute, Cary, NC).

Results

Expression Cassette Optimization. To optimize the pDNA expression cassette for PA and LF, five parameters were evaluated: (*i*) full-length vs. subdomains; (*ii*) wild-type nucleotide sequence vs. human codon-optimized sequence; (*iii*) wild-type-deduced

Table 1. Rabbit vaccination experimental groups

Group	pDNA injected	*n*	Vaccination, weeks
1	PA83Δfurin	10	0, 4, 8
2	PA83Δfurin DMRIE/DOPE	10	0, 4, 8
3	PA83Δfurin	10	0, 4
4	PA83Δfurin	10	0, 4, 8
5	PA83Δfurin plus LF[I]	10	0, 4
6	PA83Δfurin plus LF[I–III]	10	0, 4, 8
7	LF[I–III]	10	0, 4, 8
8	Vector	5	0, 4, 8
9	AVA	4	4, 8

Rabbits were injected with pDNA (0.5 mg/0.5 ml per leg) formulated with Vaxfectin (groups 1 and 3–8), or DMRIE/DOPE (group 2). Group 9 animals were vaccinated with 50 liters of AVA diluted to 0.5 ml in PBS. Group 4 rabbits were monitored monthly to assess the duration of the immune response and long-term protection.

amino acid sequence vs. sequence deleted of predicted N-linked glycosylation sites; (*iv*) deduced amino acid sequence with vs. without predicted enzymatic processing or active sites; and (*v*) deduced amino acid sequence with vs. without predicted multimerization or conformational-dependent domains. In all, six PA pDNAs and seven LF pDNAs were constructed (Fig. 4, which is published as supporting information on the PNAS web site). Comparison of pDNAs by *in vitro* expression assays of transfected mammalian cells and by immunogenicity assays (ELISA and Letx neutralization) of serum from mice injected i.m. with formulated pDNA (Table 3 and Fig. 5, which are published as supporting information on the PNAS web site) indicated that a codon-optimized, noncleavable, full-length PA (PA83Δfurin) and either of two truncated LF (LF[I] or LF [I–III]) expression plasmids were worthy of further evaluation in rabbits, a well accepted model to test immunogenicity and protection induced by candidate anthrax vaccines (14). A 150-rabbit study was conducted to test the immunogenicity of PA and LF pDNAs, combinations of PA and LF pDNAs, and delivery using several formulations. The portion of the study that was advanced to aerosolized inhalation spore challenge is presented here.

Confirmation in Rabbits of *in Vivo* Biological Activity of the Expression Cassette. Total anti-PA and anti-LF antibody titers and Letx neutralizing antibody titers were used to confirm the immunogenicity of the PA and LF expression cassettes, respectively. Groups of rabbits (n = 10) received two or three monthly injections of cationic lipid-formulated PA, either alone (Table 1, groups 1–4) or in combination with one of two LF pDNAs (groups 5 and 6). All six groups achieved end-point antibody titers to PA in the range of 10^5 to 10^6. Rabbits injected with a Vaxfectin-formulated LF pDNA, either alone (group 7), or in combination with PA pDNA (groups 5 and 6), generated similarly high anti-LF antibody titers (10^5 to 10^6) (Fig. 1*B*). Formulated vector pDNA-injected animals (group 8) had no detectable antibody titers to either PA or LF (data not shown). Together, these data indicate that both the PA and the LF pDNAs generate anti-PA and anti-LF antibody responses, respectively, when injected alone (1 mg) or coinjected (0.5 mg per plasmid). Furthermore, coinjection of PA and LF pDNAs does not cause detectable interference in the immunogenicity of either of the pDNAs (e.g., for PA, compare Fig. 1*A*, groups 1 and 6; for LF, compare Fig. 1*B*, groups 6 and 7).

The geometric mean anti-PA titers of 10^5 in rabbits after two injections with formulated PA pDNA were comparable to anti-PA titers in the four animals (group 9) given two 50-μl injections of AVA previously shown to protect rabbits from aerosolized spore challenge (19). Indeed, the anti-PA and anti-

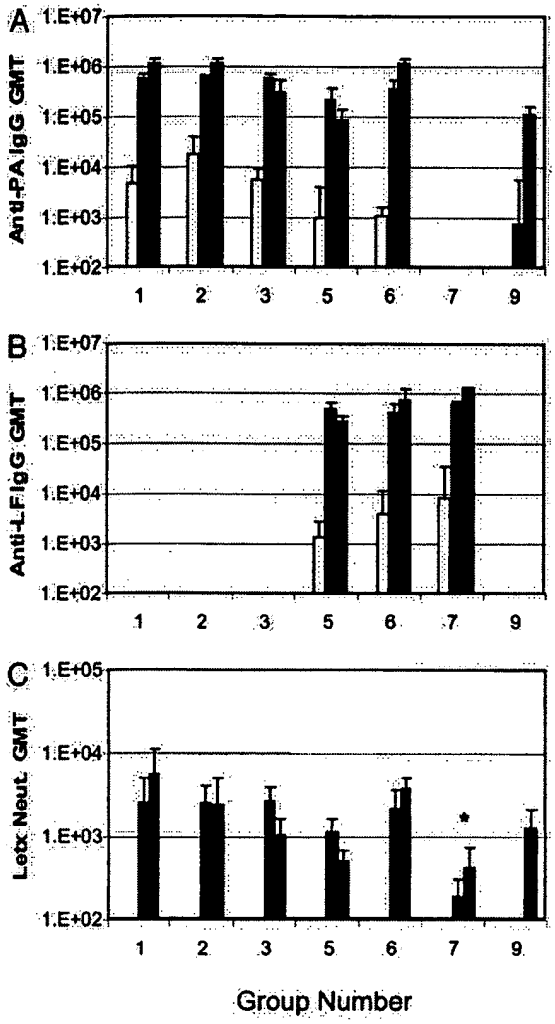

Fig. 1. Immune responses in vaccinated rabbits. Shown are group geometric mean titers (GMT) at 2 weeks (□), 6 weeks (▨), and 10 weeks (■). Shown are anti-PA IgG titer (*A*), anti-LF IgG titer (*B*), and Letx neutralization titer (*C*). Group 9 (AVA) was vaccinated on weeks 4 and 8; therefore, the 10-week value is 2 weeks after the second vaccination, an equivalent time point to the 6-week values for groups 1–8. No titer was detected in vector-immunized rabbits (group 8). *, Group 7 (LF[I-III]) was statistically lower than all other groups given three injections (groups 1, 2, and 6).

LF titers and the Letx neutralization titers were sufficiently high after two injections to justify forgoing the third injection in two groups of animals (group 3, PA pDNA alone; and group 5, PA pDNA plus LF[I] pDNA) to compare the protective immune response generated after 2 injections of formulated pDNAs with two injections of AVA.

The anti-PA titers in groups 3 and 5 and the anti-LF titer in group 5 declined slightly, but not significantly, 6 weeks after the second injection (Fig. 1 *A* and *B*). In agreement with a previous cationic lipid-formulated pDNA study (18), the third DNA dose did not increase the anti-PA or -LF titers significantly, suggesting that 0.5 mg of PA or LF pDNA maximizes the response in these rabbits after two injections.

Letx neutralizing antibody titers were measured in serum from all animals after the second and third (where relevant) injections by using the J774A.1 cytotoxicity assay (12). Similar to total binding antibody titers, all rabbits injected with PA pDNA alone or in combination with LF pDNA achieved significant neutral-

Table 2. *B. anthracis* inhalation spore challenge results

Group	Vaccination	Survival	Prechallenge Letx neut titer, median ± SD	Inhaled spore dose, median LD_{50}* ± SD (LD_{50} Range)	Days to death, median ± SD
1	PA83Δfurin	8/8	5120 ± 6110	98 ± 28 (56–129)	>21
2	PA83Δfurin/DM-DP	8/8	2560 ± 3055	116 ± 69 (56–238)	>21
3	PA83Δfurin, 2 inj.	8/8	1076 ± 634	94 ± 74 (52–252)	>21
4†	PA83Δfurin	10/10	844 ± 843	93 ± 40 (27–155)	>21
5	PA83Δfurin plus LF[I], 2inj.	8/8	415 ± 199	95 ± 43 (72–205)	>21
6	PA83Δfurin plus LF[I–III]	8/8	3948 ± 1325	113 ± 47 (65–192)	>21
7	LF[I–III]	5/9	453 ± 339	112 ± 67 (46–241)	5.3 ± 1.5
8	Vector	0/5	<20	90 ± 35 (58–144)	2.6 ± 0.5
9	AVA, 2 inj.	4/4	1280 ± 805	135 ± 34 (107–176)	>21
	Naive rabbits	0/12	ND	110 ± 49 (57–208)	2.6 ± 0.7
†	Naive rabbits	0/5	ND	112 ± 72 (34–198)	2.8 ± 1.4

Rabbits were selected for challenge on week 13 by calculating the mean Letx neutralization (neut) titer for each group and excluding one rabbit with a higher and lower titer than the mean but always including the highest and lowest titer animals. DM-DP, DMRIE-DOPE; inj, injections.

*1 LD_{50} = 105,000 Ames spores.

†All 10 group 4 rabbits and five naive animals were challenged on week 40.

izing antibody titers after two injections (Fig. 1*C*) that were similar to the titers achieved after two injections of AVA. These neutralizing titers were minimally boosted with a third injection of PA pDNA and/or LF pDNA. Only a moderate decline in Letx neutralizing antibody titers was observed at the later time points in groups 3 and 5 given only two injections of pDNA. Animals administered LF pDNA alone achieved a group geometric mean Letx neutralization titer significantly below all of the other pDNA groups given three injections, suggesting that the high titer of anti-LF binding antibody in these animals was not as effective as anti-PA antibodies in neutralizing Letx (Fig. 1).

Demonstration of Protective Immunity by Aerosolized Inhalation Challenge. Eight rabbits selected from groups 1–3, 5, and 6, nine rabbits from group 7, five blank vector-injected rabbits (group 8), all four AVA-vaccinated rabbits (group 9), and 12 naive animals were subjected to an inhalation challenge targeted at 100× the rabbit LD_{50} aerosolized spores ≈5 weeks after the third injection (study day 90+). All 40 rabbits vaccinated with PA pDNA, either alone (groups 1, 2 and 3) or in combination with LF pDNA (groups 5 and 6) survived challenge (Table 2), including all 16 animals that received only two injections of pDNA. The four rabbits vaccinated twice with AVA also survived. None of the 12 naive rabbits and none of the five rabbits injected with formulated vector pDNA (group 8) survived challenge. Protection was also achieved in five of the nine animals injected three times with LF pDNA (group 7), demonstrating that an immune response to LF does confer protection, but not as complete as the protection elicited by PA immunization. The average time to death was 5.3 days for the four nonsurviving rabbits in group 7, which, when compared with the 2.6-day average survival of control animals (P = 0.035), further suggests that LF provided measurable, although incomplete, protective immunity.

Immune Responses After Exposure to Pathogen. To determine the effect of spore challenge, serum antibodies were assayed in all surviving rabbits 7 and 21 days postchallenge (Fig. 2). Spore challenge boosted the mean anti-PA antibody titer >10-fold in AVA-vaccinated rabbits (group 9) and 2–3 orders of magnitude (P = 0.035) in surviving rabbits vaccinated with LF pDNA alone (Fig. 2*A*, group 7). This result is consistent with the germination of spores in these rabbits resulting in sufficient Letx production to boost their anti-PA titer. In contrast, rabbits vaccinated with two or three injections of PA pDNA alone (groups 1, 2, and 3) or in combination with LF pDNA (groups 5 and 6) showed no significant increase in anti-PA antibody titers postchallenge (Fig. 2*A*).

With respect to anti-LF antibody responses, AVA-vaccinated animals (group 9) developed a significant anti-LF antibody titer postchallenge (P = 0.038), again consistent with the germination of spores in these rabbits (Fig. 2*B*). Importantly, no measurable anti-LF antibody titer was induced postchallenge in animals vaccinated three times with PA pDNA alone (Fig. 2*B*, groups 1 and 2). Even in rabbits immunized only twice with PA pDNA (group 3), the minimal anti-LF titer seen on day 21 postchallenge was due to a response in just two of the eight rabbits. No significant increases in postchallenge anti-LF titers were observed in rabbits vaccinated with LF pDNA (Fig. 2*B*, groups 5 and 6, and survivors in group 7).

A final indication of the magnitude and quality of the immune response generated with pDNA is that no significant increases in postchallenge Letx neutralizing antibody titers were observed in any pDNA-vaccinated rabbits. In contrast, a significant increase (P = 0.043) in neutralizing antibody titer was seen for the four AVA-vaccinated rabbits (group 9) 21 days postchallenge.

Duration of Protective Immunity. To determine the kinetics and durability of the immune response, group 4 rabbits injected three times with Vaxfectin-formulated PA pDNA were not challenged at week 12, but rather were monitored for an additional 7 months before spore challenge. Anti-PA and Letx neutralization titers were measured monthly and showed similar profiles (Fig. 3): titers peaked at week 10 (2 weeks after the third injection) and then declined somewhat until week 26. From week 26 to the time of challenge, the titers were essentially unchanged. At week 40, all 10 rabbits were challenged as previously described and survived, whereas all five naive animals died (Table 2). In contrast to the eight group 1 rabbits that were vaccinated three times with Vaxfectin-formulated PA pDNA and challenged at week 12, spore challenge induced a significant increase in the Letx neutralization titer (Fig. 3, P = 0.024) in the group 4 rabbits, suggesting that there was limited spore germination after challenge in these animals. This postchallenge increase in Letx neutralization titer, however, was smaller than the increase seen in AVA- (group 9) or LF pDNA-vaccinated (group 7) rabbits challenged at week 12 (Fig. 2*C*).

Discussion

This study demonstrates that i.m. injection of cationic lipid-formulated pDNA vaccines directed against *B. anthracis* Letx

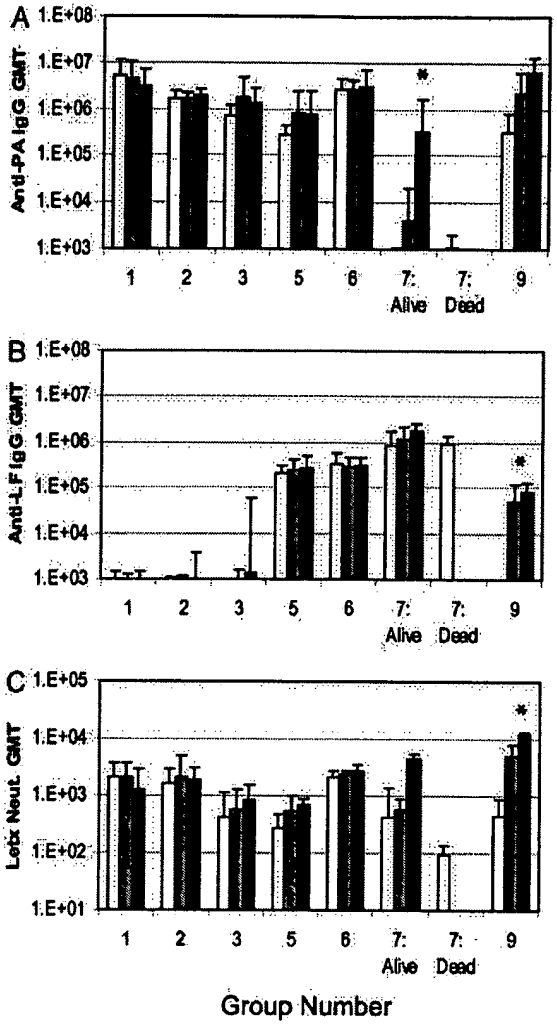

Fig. 2. Immune responses in rabbits post-anthrax spore challenge. GMTs for pDNA and AVA vaccinated rabbits pre- (□), 1-week post- (▨), and 3-weeks post- (■) *B. anthracis* inhalation spore challenge. Shown are anti-PA IgG titers (*A*), anti-LF IgG titers (*B*), and Letx neutralization titers (*C*). The GMT of the five rabbits in the LF[I-III] group that survived challenge are graphed separately from the four nonsurvivors. *, Statistically significant increase in 3-week postchallenge titers.

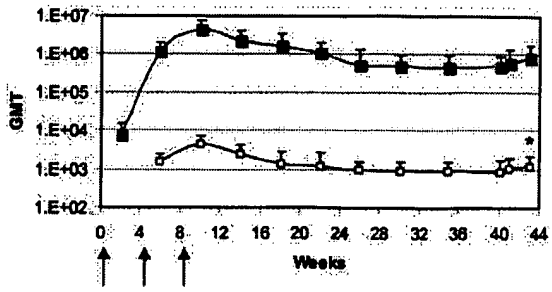

Fig. 3. Long-term persistence of immune response and postchallenge response. Group 4 rabbits were vaccinated with PA pDNA (designated by arrows) and monitored for anti-PA (■) and Letx neutralization (□) GMT. All rabbits were challenged on week 40 (32 weeks after the last immunization) and survived.

protect rabbits from a 100× LD_{50} aerosolized anthrax spore challenge. Aerosolized spore challenge is the gold standard for anthrax vaccine efficacy because it exposes the animal to the agent and expected mode of delivery anticipated in the event of a bioterrorist attack. Although there may be advantages for mucosal vaccines to be delivered by other routes (20), our data clearly show that i.m. delivered pDNA vaccines can protect against pathogenic spore exposure across a mucosal surface. Protective immunity was achieved with either two or three injections of formulated pDNA encoding detoxified PA or PA plus LF. This study also demonstrates in a relevant animal model that vaccination with LF pDNA alone can generate protective immunity. In addition, rabbits vaccinated with formulated PA pDNA achieved a durable immune response that protected them from lethal spore challenge 7 months postvaccination.

Formulated pDNA compared favorably with a 50-μl dose of AVA vaccine in this rabbit study. This dose of AVA is 1/10 the human dose, which we chose based on previous work demonstrating complete protection of rabbits from spore challenge (19). Analysis of pre- vs. postchallenge total binding and neutralizing antibody titers in animals receiving three doses of PA pDNA or PA plus LF pDNA suggests that pDNA, but not two 50-μl doses of AVA, provided sterile immunity. The sterile immunity seen in vaccinated animals at peak titer was not seen in animals challenged 7.5 months postvaccination, indicating that sterile immunity is a time-limited immunological state. Support for the mechanism by which sterile immunity can be achieved with anti-toxin antibodies comes from studies of opsonization of anthrax spores: anti-PA antibodies have been shown to opsonize anthrax spores, leading to their uptake and destruction in macrophages (21, 22). Rabbits having sufficient anti-PA antibodies to limit spore germination and bacterial growth would be predicted to have anti-PA, anti-LF, or Letx neutralization titers that do not change postchallenge.

Immune responses in rabbits injected twice with a 50-μl dose of AVA (group 9), PA pDNA (group 3), or PA plus LF pDNAs (group 5) were all of a similar magnitude before challenge. However, after challenge, the AVA animals demonstrated the largest increase in anti-PA, anti-LF, and Letx neutralizing titers, suggesting that the AVA group allowed more spore germination and concomitant Letx production. This result is dramatically shown in the AVA group by the increase in mean anti-LF titers from background levels before challenge to 81,000 postchallenge. The only postchallenge increase of similar magnitude was the anti-PA response in rabbits receiving LF pDNA alone (group 7). Animals receiving three 1-mg doses of PA pDNA alone (groups 1 and 2) showed no increase in total PA binding and neutralizing antibody titers at 7 and 21 days postchallenge. In addition, these animals generated no challenge-induced antibody to LF. Similarly, in animals receiving three injections of PA plus LF pDNAs (group 6), postchallenge PA- or LF-binding antibody titers and neutralizing antibody titers did not increase. Together, these data suggest that rabbits vaccinated three times with PA pDNA-containing vaccines develop a robust protective immune response that prevented sufficient spore germination to boost preexisting antibody responses.

Rabbits that received two injections of Vaxfectin-formulated pDNA were protected from challenge. However, serum binding and neutralizing antibody titers decreased during the 8- to 9-week period after their second injection. Therefore, we measured the duration of the protective immune response generated in rabbits after vaccination with pDNA. In the 10 animals followed for >7 months after their third vaccination, binding and neutralizing antibody titers plateaued ≈3 months after the third injection and remained stable for the duration of this 7.5-month postvaccination period. At this plateau level, the immune response still protected all 10 rabbits from a 100× LD_{50} spore challenge. However, in contrast to rabbits challenged at peak

titer, a 1.7-fold significant increase in mean Letx neutralizing titer postchallenge was seen, indicating sufficient spore generation to boost the preexisting immune response in these rabbits.

Although anti-LF antibodies were less protective than anti-PA antibodies, there are several reasons to advance a bivalent PA plus LF anthrax vaccine. A vaccine able to neutralize both components of Letx may protect against genetically engineered PA- or LF-containing toxins. Additionally, a recent study (23) demonstrating that LF (without PA) is able to permeate mammalian cells is a clear indication that the unchecked release of LF into serum cannot be considered harmless. Finally, the possible added benefit provided by coinjecting the LF pDNA did not compromise the immune response generated by the PA pDNA.

The prevention of anthrax disease is likely to be augmented by both the innate and cellular arms of the immune system, in addition to a protective humoral response. DNA vaccines have been shown to induce both $CD4^+$ and $CD8^+$ T cells (24). Such responses are likely to be induced by our DNA approach and may contribute to the control of disease, especially in the host attempt to clear infected cells and macrophages. Additionally, the combination of cationic lipids and DNA has been shown to activate an innate immune response that has antitumor activity (25), and such a response in the setting of a spore challenge may be critical in tipping the balance in favor of the host during the hours and days after exposure.

To date, the antibody responses in humans vaccinated with naked pDNA-based products have not been sufficient to warrant further product development (26). The Vaxfectin and DMRIE/DOPE cationic lipid formulations used in this study have not yet been tested in humans but have been shown to significantly enhance antigen-specific antibody responses to pDNA vaccines in animals (18, 27–29), possibly by the induction of cytokines like IFN-γ, IL-12, and IL-6. The two cationic lipid formulations performed equally well in this study and offer an attractive option to exploit the utility of pDNA technology. The advantages of a pDNA vaccine approach are that (*i*) target immunogens can be genetically, and thus specifically, stably and uniformly detoxified; (*ii*) a large number of different constructs and combinations of immunogens (i.e., multivalent vaccines) can be rapidly developed and tested; (*iii*) a range of formulations suitable for use in humans can be tested that can provide the optimal balance of humoral and cellular immune responses without immunopathology; (*iv*) pDNA does not require the handling of the pathogen or any mammalian cell substrates (with the associated risk of adventitious agents) at any point in the manufacturing process; and (*v*) stable and scalable pDNA vaccines can be rapidly transitioned to the clinic, requiring only DNA sequence information and minor manufacturing process development (24).

The rabbit studies described herein are part of an anthrax vaccine development project that has progressed from plasmid design and construction through *in vivo* immunogenicity and inhalation spore challenge, resulting in product selection, preclinical safety studies, and U.S. FDA Investigational New Drug allowance. This progress has all been accomplished within a 28-month span. The uniformity of DNA vaccine technology suggests that this product development timeframe can be repeated with other biodefense vaccine targets. Therefore, DNA vaccines provide a valuable technology for the rapid development of safe and efficacious vaccines needed for biodefense and emerging infectious diseases.

We thank Alain Rolland, Larry Smith, and Alan Engbring for review of this manuscript and Margaret Hogan for help with the figures. This study was partially funded by National Institutes of Health Small Business Technology Transfer Grant 1R41AI053060-01.

1. Joellenbeck, L., Zwanziger, L. L., Durch, J. S. & Strom, B. L., eds. (2002) *The Anthrax Vaccine: Is It Safe? Does It Work?* (Natl. Acad. Press, Washington, DC).
2. Dixon, T. C., Meselson, M., Guillemin, J. & Hanna, P. C. (1999) *N. Engl. J. Med.* **341**, 815–826.
3. Mock, M. & Mignot, T. (2003) *Cell Microbiol.* **5**, 15–23.
4. Bradley, K. A., Mogridge, J., Mourez, M., Collier, R. J. & Young, J. A. (2001) *Nature* **414**, 225–229.
5. Klimpel, K. R., Molloy, S. S., Thomas, G. & Leppla, S. H. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 10277–10281.
6. Singh, Y., Chaudhary, V. K. & Leppla, S. H. (1989) *J. Biol. Chem.* **264**, 19103–19107.
7. Mogridge, J., Cunningham, K., Lacy, D. B., Mourez, M. & Collier, R. J. (2002) *Proc. Natl. Acad. Sci. USA* **99**, 7045–7048.
8. Duesbery, N. S., Webb, C. P., Leppla, S. H., Gordon, V. M., Klimpel, K. R., Copeland, T. D., Ahn, N. G., Oskarsson, M. K., Fukasawa, K., Paull, K. D. & Vande Woude, G. F. (1998) *Science* **280**, 734–737.
9. Moayeri, M., Haines, D., Young, H. A. & Leppla, S. H. (2003) *J. Clin. Invest.* **112**, 670–682.
10. Gu, M. L., Leppla, S. H. & Klinman, D. M. (1999) *Vaccine* **17**, 340–344.
11. Riemenschneider, J., Garrison, A., Geisbert, J., Jahrling, P., Hevey, M., Negley, D., Schmaljohn, A., Lee, J., Hart, M. K., Vanderzanden, L., *et al.* (2003) *Vaccine* **21**, 4071–4080.
12. Price, B. M., Liner, A. L., Park, S., Leppla, S. H., Mateczun, A. & Galloway, D. R. (2001) *Infect. Immun.* **69**, 4509–4515.
13. Galloway, D., Liner, A., Legutki, J., Mateczun, A., Barnewall, R. & Estep, J. (2004) *Vaccine* **22**, 1604–1608.
14. Zaucha, G. M., Pitt, L. M., Estep, J., Ivins, B. E. & Friedlander, A. M. (1998) *Arch. Pathol. Lab. Med.* **122**, 982–992.
15. Brossier, F., Weber-Levy, M., Mock, M. & Sirard, J. C. (2000) *Infect. Immun.* **68**, 1781–1786.
16. Hartikka, J., Sawdey, M., Cornefert-Jensen, F., Margalith, M., Barnhart, K., Nolasco, M., Vahlsing, H. L., Meek, J., Marquet, M., Hobart, P., *et al.* (1996) *Hum. Gene Ther.* **7**, 1205–1217.
17. Pannifer, A. D., Wong, T. Y., Schwarzenbacher, R., Renatus, M., Petosa, C., Bienkowska, J., Lacy, D. B., Collier, R. J., Park, S., Leppla, S. H., *et al.* (2001) *Nature* **414**, 229–233.
18. Hartikka, J., Bozoukova, V., Ferrari, M., Sukhu, L., Enas, J., Sawdey, M., Wloch, M. K., Tonsky, K., Norman, J., Manthorpe, M. & Wheeler, C. J. (2001) *Vaccine* **19**, 1911–1923.
19. Pitt, M. L., Little, S. F., Ivins, B. E., Fellows, P., Barth, J., Hewetson, J., Gibbs, P., Dertzbaugh, M. & Friedlander, A. M. (2001) *Vaccine* **19**, 4768–4773.
20. Boyaka, P. N., Tafaro, A., Fischer, R., Leppla, S. H., Fujihashi, K. & McGhee, J. R. (2003) *J. Immunol.* **170**, 5636–5643.
21. Welkos, S., Little, S., Friedlander, A., Fritz, D. & Fellows, P. (2001) *Microbiology* **147**, 1677–1685.
22. Welkos, S., Friedlander, A., Weeks, S., Little, S. & Mendelson, I. (2002) *J. Med. Microbiol.* **51**, 821–831.
23. Kushner, N., Zhang, D., Touzjian, N., Essex, M., Lieberman, J. & Lu, Y. (2003) *Proc. Natl. Acad. Sci. USA* **100**, 6652–6657.
24. Donnelly, J. J., Ulmer, J. B. & Liu, M. A. (1998) *Dev. Biol. Stand.* **95**, 43–53.
25. Horton, H. M., Dorigo, O., Hernandez, P., Anderson, D., Berek, J. S. & Parker, S. E. (1999) *J. Immunol.* **163**, 6378–6385.
26. Srivastava, I. K. & Liu, M. A. (2003) *Ann. Intern. Med.* **138**, 550–559.
27. Reyes, L., Hartikka, J., Bozoukova, V., Sukhu, L., Nishioka, W., Singh, G., Ferrari, M., Enas, J., Wheeler, C. J., Manthorpe, M. & Wloch, M. K. (2001) *Vaccine* **19**, 3778–3786.
28. Fischer, L., Tronel, J. P., Minke, J., Barzu, S., Baudu, P. & Audonnet, J. C. (2003) *Vaccine* **21**, 1099–1102.
29. D'Souza, S., Rosseels, V., Denis, O., Tanghe, A., De Smet, N., Jurion, F., Palfliet, K., Castiglioni, N., Vanonckelen, A., Wheeler, C. & Huygen, K. (2002) *Infect. Immun.* **70**, 3681–3688.

d.6. Silicon carbide;
d.7. Tantalum or tantalum alloys;
d.8. Titanium or titanium alloys;
d.9. Titanium carbide; *or*
d.10. Zirconium or zirconium alloys.
e. Distillation or absorption columns of internal diameter greater than 0.1 m, and liquid distributors, vapor distributors or liquid collectors designed for such distillation or absorption columns, where all surfaces that come in direct contact with the chemical(s) being processed are made from any of the following materials:
e.1. Alloys with more than 25% nickel and 20% chromium by weight;
e.2. Fluoropolymers;
e.3. Glass (including vitrified or enamelled coatings or glass lining);
e.4. Graphite or carbon-graphite;
e.5. Nickel or alloys with more than 40% nickel by weight;
e.6. Tantalum or tantalum alloys;
e.7. Titanium or titanium alloys; *or*
e.8. Zirconium or zirconium alloys.
f. Remotely operated filling equipment in which all surfaces that come in direct contact with the chemical(s) being processed are made from any of the following materials:
f.1. Alloys with more than 25% nickel and 20% chromium by weight; *or*
f.2. Nickel or alloys with more than 40% nickel by weight.
g. Valves with nominal sizes greater than 1.0 cm (3/8 in.), in which all surfaces that come in direct contact with the chemical(s) being processed or contained are made from any of the following materials:
g.1. Nickel or alloys with more than 40% nickel by weight;
g.2. Alloys with more than 25% nickel and 20% chromium by weight;
g.3. Fluoropolymers;
g.4. Glass or glass lined (including vitrified or enameled coatings);
g.5. Tantalum or tantalum alloys;
g.6. Titanium or titanium alloys; *or*
g.7. Zirconium or zirconium alloys.
h. Multi-walled piping incorporating a leak detection port, in which all surfaces that come in direct contact with the chemical(s) being processed or contained are made from any of the following materials:
h.1. Alloys with more than 25% nickel and 20% chromium by weight;
h.2. Fluoropolymers;
h.3. Glass (including vitrified or enamelled coatings or glass lining);
h.4. Graphite or carbon-graphite;
h.5. Nickel or alloys with more than 40% nickel by weight;
h.6. Tantalum or tantalum alloys;
h.7. Titanium or titanium alloys; *or*
h.8. Zirconium or zirconium alloys.
i. Multiple-seal, canned drive, magnetic drive, bellows or diaphragm pumps, with manufacturer's specified maximum flow-rate greater than 0.6 m^3/hour, or vacuum pumps with manufacturer's specified maximum flow-rate greater than 5 m^3/hour (under standard temperature (273 K (0° C)) and pressure (101.3 kPa) conditions), and casing (pump bodies), preformed casing liners, impellers, rotors or jet pump nozzles designed for such pumps, in which all surfaces that come into direct contact with the chemical(s) being processed are made from any of the of the following materials:
i.1. Alloys with more than 25% nickel and 20% chromium by weight;
i.2. Ceramics;
i.3. Ferrosilicon;
i.4. Fluoropolymers;
i.5. Glass (including vitrified or enamelled coatings or glass lining);
i.6. Graphite or carbon-graphite;
i.7. Nickel or alloys with more than 40% nickel by weight;
i.8. Tantalum or tantalum alloys;
i.9. Titanium or titanium alloys, *or*
i.10. Zirconium or zirconium alloys.
j. Incinerators designed to destroy chemical warfare agents, chemical weapons precursors controlled by 1C350, or chemical munitions having specially designed waste supply systems, special handling facilities and an average combustion chamber temperature greater than 1000°C in which all surfaces in the waste supply system that come into direct contact with the waste products are made from or lined with any of the following materials:
j.1. Alloys with more than 25% nickel and 20% chromium by weight;
j.2. Ceramics; *or*
j.3. Nickel or alloys with more than 40% nickel by weight.

Technical Note: Carbon-graphite is a composition consisting primarily of graphite and amorphous carbon, in which the graphite is 8 percent or more by weight of the composition.

19. In Supplement No. 1 to Part 774 (the Commerce Control List), Category 2—Materials Processing, is amended by revising the List of Items Controlled section in ECCN 2B352 to read as follows:

2B352 Equipment capable of use in handling biological materials, as follows (see List of Items Controlled).

* * * * *

List of Items Controlled

Unit: Equipment in number.
Related Controls: N/A.
Related Definitions: For purposes of this entry, isolators include flexible isolators, dry boxes, anaerobic chambers and glove boxes.
Items:
a. Complete containment facilities at P3 or P4 containment level.

Technical Note: P3 or P4 (BL3, BL4, L3, L4) containment levels are as specified in the WHO Laboratory Biosafety Manual (Geneva, 1983).

b. Fermenters capable of cultivation of pathogenic microorganisms, viruses, or for toxin production, without the propagation of aerosols, having a capacity equal to or greater than 100 liters.

Technical Note: Fermenters include bioreactors, chemostats, and continuous-flow systems.

c. Centrifugal separators capable of the continuous separation of pathogenic microorganisms, without the propagation of aerosols, and having all of the following characteristics:
c.1. One or more sealing joints within the steam containment area;
c.2. A flow rate greater than 100 liters per hour;
c.3. Components of polished stainless steel or titanium; *and*
c.4. Capable of *in situ* steam sterilization in a closed state.

Technical Note: Centrifugal separators include decanters.

d. Cross (tangential) flow filtration equipment capable of continuous separation of pathogenic microorganisms, viruses, toxins, and cell cultures without the propagation of aerosols, having all of the following characteristics:
d.1. Equal to or greater than 5 square meters;
d.2. Capable of *in situ* sterilization.
e. Steam sterilizable freeze-drying equipment with a condenser capacity of 10 kgs of ice or greater in 24 hours, but less than 1,000 kgs of ice in 24 hours.
f. Protective and containment equipment, as follows:
f.1. Protective full or half suits, or hoods dependant upon a tethered external air supply and operating under positive pressure;

Technical Note: This entry does not control suits designed to be worn with self-contained breathing apparatus.

f.2. Class III biological safety cabinets or isolators with similar performance standards, *e.g.*, flexible isolators, dry boxes, anaerobic chambers, glove boxes or laminar flow hoods (closed with vertical flow).
g. Chambers designed for aerosol challenge testing with microorganisms, viruses, or toxins and having a capacity of 1 m^3 or greater.

Dated: May 23, 2002.

James J. Jochum,

Assistant Secretary for Export Administration.

[FR Doc. 02–13581 Filed 5–30–02; 8:45 am]
BILLING CODE 3510–33–P

DEPARTMENT OF HEALTH AND HUMAN SERVICES

Food and Drug Administration

21 CFR Parts 314 and 601

[Docket No. 98N–0237]

RIN 0910–AC05

New Drug and Biological Drug Products; Evidence Needed to Demonstrate Effectiveness of New Drugs When Human Efficacy Studies Are Not Ethical or Feasible

AGENCY: Food and Drug Administration, HHS.

ACTION: Final rule.

SUMMARY: The Food and Drug Administration (FDA) is amending its new drug and biological product regulations to allow appropriate studies in animals in certain cases to provide

substantial evidence of the effectiveness of new drug and biological products used to reduce or prevent the toxicity of chemical, biological, radiological, or nuclear substances. This rule will apply when adequate and well-controlled clinical studies in humans cannot be ethically conducted and field efficacy studies are not feasible. In these situations, certain new drug and biological products that are intended to reduce or prevent serious or life-threatening conditions may be approved for marketing based on evidence of effectiveness derived from appropriate studies in animals and any additional supporting data.

DATES: This rule is effective July 1, 2002.

FOR FURTHER INFORMATION CONTACT:
Wayne H. Mitchell, Center for Drug Evaluation and Research (HFD–7), Food and Drug Administration, 5600 Fishers Lane, Rockville, MD 20857, 301–594–2041;
or Karen L. Goldenthal, Center for Biologics Evaluation and Research (HFM–475), 1401 Rockville Pike, suite 370 North, Rockville, MD 20852, 301–827–3070.

SUPPLEMENTARY INFORMATION:

I. Introduction

In the **Federal Register** of October 5, 1999 (64 FR 53960), we (FDA) proposed to amend our new drug and biological product regulations to identify the information needed to provide substantial evidence of the effectiveness of certain new drug and biological products used to reduce or prevent the toxicity of chemical, biological, radiological, or nuclear substances. We are finalizing that proposed rule by adding subpart I to part 314 (21 CFR part 314) and subpart H to part 601 (21 CFR part 601).

This final rule provides for approval of certain new drug and biological products based on animal data when adequate and well-controlled efficacy studies in humans cannot be ethically conducted because the studies would involve administering a potentially lethal or permanently disabling toxic substance or organism to healthy human volunteers and field trials are not feasible prior to approval. Under this rule, in these situations, certain new drug and biological products that are intended to reduce or prevent serious or life-threatening conditions can be approved for marketing based on evidence of effectiveness derived from appropriate studies in animals, without adequate and well-controlled efficacy studies in humans (§ 314.126). In assessing the sufficiency of animal data, the agency may take into account other data, including human data, available to the agency. Under this rule, FDA can rely on the evidence from animal studies to provide substantial evidence of the effectiveness of these products when:

1. There is a reasonably well-understood pathophysiological mechanism for the toxicity of the chemical, biological, radiological, or nuclear substance and its amelioration or prevention by the product;

2. The effect is demonstrated in more than one animal species expected to react with a response predictive for humans, unless the effect is demonstrated in a single animal species that represents a sufficiently well-characterized animal model (meaning the model has been adequately evaluated for its responsiveness) for predicting the response in humans;

3. The animal study endpoint is clearly related to the desired benefit in humans, which is generally the enhancement of survival or prevention of major morbidity; and

4. The data or information on the pharmacokinetics and pharmacodynamics of the product or other relevant data or information in animals and humans is sufficiently well understood to allow selection of an effective dose in humans, and it is therefore reasonable to expect the effectiveness of the product in animals to be a reliable indicator of its effectiveness in humans.

All studies subject to this rule must be conducted in accordance with preexisting requirements under the good laboratory practices (21 CFR part 58) regulations and the Animal Welfare Act (7 U.S.C. 2131 *et. seq.*).

Safety evaluation of products is not addressed in this rule. Products evaluated for effectiveness under subpart I of part 314 and subpart H of part 601 will be evaluated for safety under preexisting requirements for establishing the safety of new drug and biological products. The agency believes that the safety of most of these products can be studied in human volunteers similar to the people who would be exposed to the product. FDA recognizes that some safety data, such as data on possible adverse interactions between the toxic substance itself and the new product, may not be available. This is not expected to keep the agency from making an adequate safety evaluation. FDA's procedures and standards for evaluating the safety of new drug and biological products are sufficiently flexible to provide for the safety evaluation of products evaluated for efficacy under subpart I of part 314 and subpart H of part 601.

This rule will not apply if product approval can be based on standards described elsewhere in our regulations (for example, accelerated approval based on human surrogate markers or clinical endpoints other than survival or irreversible morbidity).[1]

II. Comments on the Proposed Rule and Our Response

We received comments on the proposed rule from two pharmaceutical companies and one physician affiliated with a university. We also received comments from the National Institutes of Health (NIH). The NIH comments were based on a prepublication draft of the proposed rule, but the comments were received too late to be addressed in the proposed rule. The NIH comments have been placed in the docket for this rule and are addressed in this document.

In addition to the changes we have made in response to comments, we have changed the titles of subpart I of part 314 and subpart H (formerly subpart G) of part 601 to better describe the scope of the subparts. Subpart I of part 314 is now entitled "Approval of New Drugs When Human Efficacy Studies Are Not Ethical or Feasible" and subpart H of part 601 is now entitled "Approval of Biological Products When Human Efficacy Studies Are Not Ethical or Feasible." Proposed subpart G has been redesignated as subpart H in the final rule because subpart G has since been designated for regulations on postmarketing studies. Proposed §§ 601.60 through 601.65 have been renumbered §§ 601.90 through 601.95 in subpart H.

We have also changed, on our own initiative, the requirements proposed in §§ 314.610(c) and 601.61(c) (§§ 314.610(b)(3) and 601.91(b)(3) in this final rule). We have deleted the requirement that self-administered drug products approved under this rule be in unit-of-use packaging with attached patient labeling. In addition, we have eliminated the distinction between self-

[1] An example of a drug approval based on human surrogate markers is our August 30, 2000, approval of an efficacy supplement for ciprofloxacin. Ciprofloxacin HCl was approved for postexposure management of inhalational anthrax. The approval was based, in part, on human studies demonstrating that ciprofloxacin achieved serum concentrations reaching or exceeding levels associated with improved survival of animals exposed to aerosolized *Bacillus anthracis* spores. The results from these studies were combined with the knowledge of effectiveness in humans of ciprofloxacin for other bacterial infections, including pneumonia. The validity of the human surrogate marker was supported by animal studies.

Development of anthrax DNA vaccines

Marilyn E Ferrari, Gary Hermanson & Alain Rolland*

Address
Vical Inc
10390 Pacific Center Court
San Diego
CA 92121
USA
Email: arolland@vical.com

*To whom correspondence should be addressed

Current Opinion in Molecular Therapeutics 2004 6(5):506-512
 ISSN 1464-8431

Over 120 years ago, Pasteur and Greenfield developed an in vitro procedure for producing a live-attenuated Bacillus anthracis bacterial culture capable of protecting livestock from anthrax disease. Since then, anthrax has become one of the best characterized bacterial pathogens with regard to mechanism of toxicity and vaccine development. Most developments have used live-attenuated strains, bacterial supernatants or protein subunit approaches. Recently, novel plasmid DNA (pDNA) approaches to a safe and effective anthrax vaccine have been proposed. This review summarizes the history of anthrax, the need for new vaccines and recent developments in pDNA-based vaccines, leading to the initiation of a human phase I clinical trial in a significantly shorter timeframe than in traditional vaccine development.

Keywords Anthrax, *Bacillus anthracis*, DNA vaccine, lethal factor, lethal toxin, plasmid-based vaccine, protective antigen

Introduction

History of anthrax

Bacillus anthracis, the causative agent of anthrax, is a Gram-positive, spore-forming bacterium. The spores are extremely stable in adverse conditions, and are resistant to heat, ultraviolet light, pressure and chemical agents. The disease primarily infects herbivores, however, all mammals are susceptible to anthrax infection [1••].

The incidence of naturally occurring anthrax in humans is fairly rare, with a risk of infection of approximately 1/100,000 [2]. There are three main forms of the disease: cutaneous, gastrointestinal and inhalation. Cutaneous is the most common form, accounting for 95% of all US cases. There have been no reported US cases of the gastrointestinal form (which is generally due to poorly cooked meat), and inhaled anthrax (also called woolsorter's disease) is rare, although usually fatal [3]. Prior to the anthrax attacks of 2001 in the US, cases of inhalational anthrax were limited. The first incident occurred in Sverdlovsk, Russia, following the accidental release of spores from a Soviet bioweapons facility. There were 18 occupational cases in the US in the 20th century. Most of these cases occurred among mill or tannery workers, and two were associated with laboratory workers [4]. Recently, however, there have been reports of fatal respiratory illnesses attributed to *Bacillus cereus*. Thus, it appears that other *Bacillus* species are capable of causing a disease similar to the inhalation form of the anthrax disease [5].

Anthrax virulence factors

The virulence of *B anthracis* in rabbits, non-human primates and humans is primarily the result of a multicomponent toxin secreted by the organism [3]. The toxin consists of three separate proteins, protective antigen (PA), lethal factor (LF) and edema factor (EF), which are encoded on a 184-kb plasmid designated pXO1 [6•]. PA83 (735 amino acids, molecular weight of 82,684 Da) is a single-chain protein that binds to a mammalian ubiquitous cell surface receptor [7]. Cleavage by furin (or a furin-like enzyme) results in a 63-kDa receptor-bound product [8,9] that multimerizes and can bind both the 90-kDa LF protein and the 89-kDa EF protein, which are subsequently endocytosed as a complex [10]. LF (776 amino acids, molecular weight of 90,237 Da) is a zinc metalloprotease that, once internalized and released from the endosomes, cleaves several isoforms of mitogen-activated protein kinase kinase (Mek1, Mek2 and MKK3), thus disrupting signal transduction events within the cell [11]. The complex of LF and PA is referred to as lethal toxin (Letx), and is responsible for the rapid lethality of anthrax spore inhalation infection [3,12] due to extensive tissue hypoxia accompanied by pleural edema [12].

Anthrax as a biothreat and the need for a new vaccine

Anthrax mainly infects livestock and is transmitted through food and soil. Although only a small number of animals were used for the experiments, Greenfield was the first to develop an anthrax vaccine that generated protective immunity in cattle [13]. Subsequently, a protective vaccine for livestock using an attenuated *B anthracis* strain was developed by Pasteur in 1881 [14•]. Later, a live-spore vaccine was developed by Sterne [14•]; this remains in widespread use today as an effective livestock vaccine, but is considered unsafe for human use. Concern over the safety of live-attenuated bacterial vaccines for humans led to the development of anthrax vaccine adsorbed (AVA), now called BioThrax (BioPort Corp). Prior to the 1990s, BioThrax was used primarily to protect workers with an occupational risk of anthrax exposure [15]. This vaccine consists of a cell-free filtrate of *B anthracis* adsorbed to aluminum hydroxide and is the only anthrax vaccine licensed for human use in the US.

The Japanese were the first to use anthrax spores as a weapon in Manchuria during the 1940s [3]. More recently, both the former Soviet Union and Iraq acknowledged producing weaponized anthrax in 1995 [4]. In response to the threat of anthrax being used as an agent of bioterrorism, the US Department of Defense began a mandatory AVA vaccine program in 1998 to protect its military forces [15]. AVA has remained essentially unchanged since the 1960s and has been administered primarily to workers involved in processing animal products and to military personnel [16].

Several concerns with the currently licensed anthrax vaccine that have been discussed are, lot-to-lot variability, use limited to adults aged 18 to 65, a less than optimal dosing regimen from the standpoint of compliance and convenience (six doses over 18 months, followed by an annual booster), and reactogenicity (especially in women) [17]. These concerns led the Institute of Medicine to recommend the development of a second-generation anthrax vaccine [18].

The anthrax letter attacks of 2001 heightened public concern of the threat of anthrax as a bioterrorism agent. Cases of anthrax (n = 22; 11 inhalation and 11 cutaneous) were reported as a result of spores sent via the US Postal Service in 2001, with five fatalities resulting from the inhalation form of the disease [19]. These biological attacks following the September 11, 2001 terrorist attack resulted in a heightened awareness of bioterrorism threats, and the US government responded with several measures to stimulate research and development of protective agents. In addition, the National Institute of Allergy and Infectious Diseases (NIAID) initiated a series of integrated programs for biodefense research [20].

There was an overwhelming international response to the government's call for new defenses against bioterrorism from both the private and public sectors. Since September 11, 2001, there has been significant grant funding for new vaccine technologies, with over 30 grants awarded for anthrax vaccines alone [21]. In 2003 and 2004, US $437 million were allocated for anthrax research. The National Institutes of Health (NIH)'s estimated budget for biodefense research is US $1.6 billion in 2004, with the highest proportion allocated to vaccine development [14•]. The NIH issued a Request for Proposal in March 2003 for the production and testing of a recombinant PA (rPA) vaccine (PA protects against spore challenge and is the main active component of AVA [22]). rPA vaccines have been studied for many years and were the most readily available option for a second-generation vaccine.

In addition to AVA and rPA, the government is supporting new vaccine technologies. In 2004, Congress budgeted US $5.6 billion over the next ten years under Project Bioshield to procure stockpiles of bioterrorism countermeasures and encourage the development of new vaccines [23]. The Act was signed by President Bush on July 21, 2004, after receiving House and Senate approval.

In contrast to conventional or recombinant protein vaccines, plasmid DNA (pDNA) vaccination involves delivering a plasmid encoding the antigen(s) of interest to cells. The antigen is then expressed *de novo*, resulting in the induction of cellular and humoral immune responses. Plasmid-based vaccines offer many potential advantages that would make them ideal candidates as new vaccines against anthrax and other bioterrorism targets. These include: (i) the target immunogens can be genetically, and thus specifically, stably and uniformly detoxified; (ii) a large number of different constructs and combinations of immunogens (ie, multivalent vaccines) can be rapidly developed and tested; (iii) a range of formulations suitable for use in humans can be tested that can provide the optimal balance of humoral and cellular immune responses without immunopathology; (iv) pDNA does not require the handling of the pathogen or any mammalian cell substrates (with the associated risk of adventitious agents) at any point in the manufacturing process; and (v) the uniformity of testing and manufacturing regardless of the sequence of the encoded antigen, and the scalability of pDNA manufacturing facilitates the rapid advancement of pDNA vaccines from the research laboratory to the clinic [24]. The ability to develop multivalent (multi-target or multi-agent) vaccines with broad protection may also be a key advantage for biodefense.

Anthrax DNA vaccine studies

In the first published anthrax DNA vaccine study, Dennis Klinman's laboratory at the US Food and Drug Administration (FDA) demonstrated that pDNA vaccination could protect mice from an intravenously administered Letx challenge [25••]. These investigators used a pDNA encoding PA63 (with the bacterial leader sequence deleted) fused to a human tissue plasminogen activator (htPA) signal sequence to direct efficient secretion of the antigen from mammalian cells. PA63 is the biologically active component that forms the heptameric structure cleaved from the full-length 83-kDa PA protein. The study demonstrated that an anti-PA antibody response could be elicited after a single intramuscular injection of pDNA and that this response could be boosted by repeated vaccinations. Immunized mice developed a strong anti-PA antibody response that also neutralized Letx activity in an *in vitro* cytotoxicity assay. The elicited immune response was biologically relevant, as it protected all mice from an intravenous (iv) challenge of approximately 5-fold the LD_{50} of Letx.

Subsequent studies on pDNA vaccines against anthrax were expanded to use particle-mediated epidermal delivery (gene gun), boosting with recombinant protein or different routes of administration (eg, mucosal immunization). A study published in the same year as the Klinman study examined the use of pDNA alone or in combination with an rPA protein boost [26]. In an immunization schedule requiring almost a year, low levels of anti-PA antibody titers were produced with pDNA alone, but titers could be elevated after boosting with rPA. The pDNA construct encoded a full-length PA gene (including the bacterial signal sequence) without a mammalian signal sequence necessary for efficient secretion of the PA protein from the injected mouse muscle cells. Secretion of the pDNA-encoded antigen was previously demonstrated to be important for generating a strong humoral response to ovalbumin (ova) in a study comparing the immune response to ova expressed as a secreted, cytoplasmic or transmembrane protein [27]. Despite the lack of secretion, enough pDNA-expressed protein entered the major histocompatibility complex class II pathway in muscle cells or antigen-presenting cell cross-presentation pathway to prime a response that could be boosted with recombinant protein.

Although antibodies directed against PA alone were sufficient to protect mice and rabbits against a Letx challenge, some studies suggested that EF and/or LF could also contribute to protective immunity. The ability of an LF immune response to augment the protective response to PA or to offer protection on its own was tested with pDNA

delivered by a gene gun [28••]. pDNA encoding non-secreted forms of PA63 and the N-terminal 254-amino-acid fragment of LF that binds PA63 were used to immunize mice. Anti-PA and anti-LF antibodies were generated and could be boosted with recombinant protein. Importantly, all of the PA and PA + LF pDNA-immunized mice were protected from an iv challenge of approximately 5-fold the LD_{50} of Letx; in addition, all LF-only pDNA immunized mice were protected. This study was the first direct demonstration that LF provides protective efficacy.

In a follow-on study, the non-secreted PA and LF pDNAs were co-injected into three rabbits and compared with two rabbits injected with LF pDNA alone, and single rabbits injected with PA + LF, PA or LF pDNA twice followed by a recombinant protein boost. Single rabbits injected with either rPA, rPA + rLF or AVA were also included in the study [29]. Animals were challenged from 64 to 411 days later with inhaled aerosolized anthrax spores. None of the rabbits immunized with the pDNA alone survived challenge. The DNA prime with rPA and rPA + rLF boost rabbits both survived, as did all recombinant protein-immunized control rabbits. The variable immunization and challenge protocols, coupled with the low number of rabbits per group, makes analysis of the data in this study difficult. This paper is the first published example of aerosolized spore challenge of pDNA-immunized rabbits, which is the gold standard for anthrax vaccine efficacy since it exposes the animal to the agent and to the expected mode of delivery in the event of a bioterrorist attack. Inhalation challenge of rabbits is also anticipated to be an important model for vaccine approval by the FDA according to the 'animal rule' (21CFR601 subpart H), which may be needed for FDA approval of biodefense and other vaccines for which human efficacy testing is not ethical or feasible [30•].

Although a recombinant protein boost increased the antibody responses of pDNA vaccines, it is not practical for large-scale development and distribution of vaccines for biodefense. In addition, it obviates many of the key advantages of pDNA vaccines described above. The ability to conveniently deliver more than one antigen is one of the major advantages of pDNA over recombinant proteins, and was demonstrated using a five-pDNA vaccine encoding genes for both blood-stage and liver-stage antigens of *Plasmodium falciparum* [31•]. Riemenschneider *et al* extended this paradigm for bioterrorist vaccine development by testing a cocktail of four pDNAs encoding antigens from *B anthracis*, and Ebola, Marburg and Venezuelan equine encephalitis viruses [32•]. In this study, rabbits were immunized with a pDNA encoding full length PA83 fused to the htPA leader sequence for efficient secretion of the antigen. Groups of ten rabbits were immunized four times with approximately 20 µg of PA83 pDNA divided over eight sites on the skin at 0, 4, 8 and 21 weeks using the gene gun. The immune responses in these rabbits were compared directly to those in rabbits receiving the human dose of AVA (0.5 ml) four times at 0, 4, 8 and 12 weeks. Titers of anti-PA antibodies and Letx neutralization were equivalent in the pDNA and AVA rabbits, indicating that pDNA could induce the same high level of response as AVA in rabbits. Importantly, these rabbits were challenged by subcutaneous injection of 100-fold the LD_{50} of Ames strain spores and monitored for survival. While all control animals died, nine out of ten PA83 pDNA-vaccinated animals and seven out of ten AVA-vaccinated animals survived challenge, illustrating the protective efficacy of pDNA vaccines against anthrax in mammals.

Gene gun delivery has been successfully used to generate humoral responses in large animals, including humans, using microgram doses of DNA [33,34]. However, the scale-up of that technology still presents several technical hurdles that will need to be overcome for commercialization.

An alternative method of delivery for pDNA vaccines has been tested in the anthrax model. A full-length PA gene was cloned into a cytomegalovirus promoter-driven plasmid and delivered to the salivary gland by a process termed retroductal gene transfer [35]. Presumably the PA gene was not engineered to be secreted by mammalian cells by the addition of a leader sequence. A single injection of 175 µg of pDNA, formulated with a cationic lipid/co-lipid combination and Zn^{2+}, was delivered to the salivary gland of rats and compared to a single dose of intramuscularly administered pDNA and 15 µg of rPA protein in complete Freund's adjuvant. Using this delivery system, previous research had demonstrated that a second boost injection of pDNA did not increase antibody titers, due to 'the strong adaptive immune response to the encoded protein' [35], thus no boost was attempted in this study. A 10-fold minimal lethal dose of Letx was injected intravenously, and animals were monitored for survival. Four out of six salivary gland-injected animals and both rPA-vaccinated animals survived, whereas none of the six intramuscularly immunized animals survived. It is important to note that DNA was administered in combination with lipid and Zn^{2+} for salivary gland delivery, but not for intramuscular (im) delivery. It cannot be ruled out that this difference in formulation may account for the differences in survival observed between the two groups. Given the limited response of the animals to a single injection, it is difficult to understand why the immune response cannot be boosted by administering a second dose. This inability to boost the primary immune response using retroductal gene transfer coupled with administration 'in an office setting by a trained professional in less than 20 min, not unlike a dental check-up' [35] severely limits the utility of this method for prophylactic vaccination.

Due to the limitations of the approaches described above, there remains a need for improved formulations and methods of generating humoral responses with DNA vaccines. This is particularly true in biodefense indications where stability, distribution and ease of administration are key concerns. Formulation of DNA with cationic lipids has been shown in a variety of *in vivo* studies for several infectious disease targets to result in enhanced humoral responses against antigen targets. These studies are summarized in Table 1. Although it has yet to be demonstrated in humans, there is extensive preclinical evidence suggesting that cationic lipid-based formulations

Table 1. Cationic lipids used for infectious disease vaccine indications.

Lipids	Route of administration	Animal model	pDNA-encoded antigen	Dose	Reference
Vaxfectin (Vical Inc)	im	Mice	NP (flu)	Two to three administrations at varied doses (1 to 25 μg)	[39•]
	im	Rabbit	NP (flu)	Two administrations of 150 μg	[39•]
	im	Mice	Five antigens (including NP)	Two administrations of 5 μg	[40•]
	im	Mice	JE prM and E	Three administrations at varied doses (5 to 100 μg)	[41]
	im, id and in	Mice	gp140 (HIV)	Two administrations of 20 μg	[42]
	im, id and in	Baboons	Tat, Nef, Gag/Pro and Env (HIV)	Four administrations of 7 mg	[43]
Vaxfectin, GAP-DLRIE:DOPE	salivary	Mice	NP (flu)	Two administrations of 300 μg	[44]
	im/in	Mice	Ag85A, AG85B and PStS-3 (TB)	Three to four administrations of 50 μg (im) and 20 μg (in)	[45]
DMRIE:DOPE	im	Ponies	Glycoprotein G (rabies)	Two administrations of 200 μg	[36•]
	im	Dogs	Glycoprotein HA and F (canine distemper)	Two administrations of 100 μg	[46]
DDAB:PC	im	Mice	NS3 (hepatitis C)	Three administrations of 100 μg	[47]
DC-Chol:DOPE	im, ip, in, sc and id	Mice, guinea pigs	Env/Rev (HIV)	Two administrations of 5 or 10 μg	[48]
	in	Mice	Env/Rev (HIV)	Three administrations of varied doses (1 to 10 μg)	[49]
DC-Chol:DOPE (± mannan)	in and im	Mice	Rev (HIV)	Three administrations of 2 μg	[50]
DOTMA:DOPE	im, ip and iv	Mice	NP (LCMV)	One administration of 100 μg	[51]
TM-TPS:DOPE (CellFECTIN)	id and im	Monkeys	HBsAg (HBV)	Two administrations of 400 μg	[52]
DOTAP:PC:DOPE	im	Mice	HBsAg (HBV)	Three administrations of 10 μg	[53]
DODAC:DOPE:PEG$_{2000}$C8CER	im and in	Mice	HA (Flu)	Varied dosing	[54]
DOSPER	in and oral	Mice	HA (Flu)	Two administrations of 20 μg	[55]

Vaxfectin is composed of the cationic lipid VC-1052, (±)-*N*-(3-aminopropyl)-*N,N*-dimethyl-2,3-bis(myristoleyloxy)-1-propanaminium bromide and the co-lipid 1,2-diphytanoyl-*sn*-glycero-3-phosphoethanolamine. **DMRIE** ±-*N*-(2-Hydroxyethyl)-*N,N*-dimethyl-2,3-bis(tetradecyloxy)-1-propanaminium bromide, **DOPE** 1,2 Dioleoyl-*sn*-glycero-3-phosphoethanolamine, **HA** hemagglutinin, **HBV** hepatitis B virus, **id** intradermal, **im** intramuscular, **in** intranasal, **ip** intraperitoneal, **iv** intravenous, **JE** Japanese encephalitis virus, **NP** nucleoprotein, **sc** subcutaneous, **TB** tuberculosis.

significantly enhance humoral responses of DNA vaccines. An (±)-*N*-(2-hydroxyethyl)-*N,N*-dimethyl-2,3-bis(tetradecyloxy)-1-propanaminium bromide (DMRIE):1,2 dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) formulation of a plasmid encoding human leukocyte antigen-B7 and β2 microglobulin delivered intratumorally has demonstrated safety in over 700 cancer patients. DMRIE:DOPE has resulted in significant enhancement of antirabies titers in ponies compared with naked DNA [36•]. In addition, the DMRIE:DOPE pDNA formulations are scalable and they have been manufactured as a single vial product stored as a liquid suspension at 2 to 8°C for several years [Vical Inc, unpublished data]. Thus, cationic lipid-based formulations have the potential to provide a new generation of vaccines not only for anthrax, but also for other bioterrorism, infectious disease and cancer vaccine indications.

Based on the results with DMRIE:DOPE and Vaxfectin (Vical Inc; Table 1), plasmids encoding detoxified PA and LF were formulated with these two cationic lipid systems to produce an anthrax vaccine candidate. These formulations enhanced antibody response in mice relative to naked DNA [G Hermanson, unpublished data].

In a series of preclinical studies designed with the goal of developing a safe and efficacious human anthrax vaccine, Hermanson *et al* [37••] screened six different PA pDNA and seven LF pDNA constructs for *in vitro* protein expression and mouse immunogenicity, including total antibody titers and *in vitro* Letx neutralization. All constructs included the htPA leader sequence for efficient secretion from mammalian cells in order to induce the highest possible humoral response. Constructs synthesized with codons optimized for expression in humans and constructs encoding *B anthracis* wild-type sequences were compared. Full-length PA83 (with the furin cleavage site deleted), the processed PA63, and humanized and *B anthracis* wild-type PA constructs were tested. LF constructs tested included humanized and wild-type full-length versions and truncated versions in which the metalloprotease domain had been deleted.

A humanized PA83 plasmid, and two truncated LF plasmids were chosen based on their ability to induce the highest anti-PA or anti-LF antibody titers and Letx-neutralizing titers in mice. The pDNAs were used in a rabbit (n = 150) study comparing single (PA or LF) and bivalent (PA + LF) pDNA vaccinations delivered in multiple formulations [37••]. Immunogenicity parameters were measured, and a subset of rabbits was selected for 100-fold the LD_{50} anthrax spore inhalation challenge. Surviving animals were monitored for anti-PA, anti-LF and Letx neutralization titers to assess the effect of the spore challenge on the subsequent immune response. In addition, ten rabbits were monitored 7.5 months post-vaccination to determine the duration of the pDNA-induced immune response prior to a terminal inhalation spore challenge. As a benchmark, four rabbits immunized twice with one-tenth the human dose of AVA were also included [37••].

Protective immunity was achieved in all 40 rabbits immunized with either two or three 1-mg injections of formulated pDNA encoding detoxified PA83 or PA83 + LF. All four AVA-immunized rabbits also survived challenge, while five formulated non-coding plasmid controls and 12 naive rabbits died. Significantly, five out of nine rabbits immunized with LF pDNA alone were also protected from challenge. Additionally, in the four surviving animals, the time-to-death was significantly prolonged compared with the naive controls [37••]. This is the first demonstration in a relevant animal model that vaccination with LF pDNA alone can generate protective immunity.

Formulated pDNA compared favorably with a 50 µl dose of AVA vaccine in this rabbit study. Analysis of pre- versus post-challenge total binding and neutralizing antibody titers in animals receiving three doses of PA pDNA or PA + LF pDNA showed no change in the anti-PA and anti-LF or Letx-neutralizing titers post-challenge [37••]. This suggests that pDNA, but not two 50 µl doses of AVA, provided 'sterile immunity', defined as a state of immunity that prevents sufficient spore germination to boost pre-existing antibody responses.

Ten PA83-vaccinated rabbits were followed for 7.5 months after their third vaccination. Both binding and neutralizing antibody titers were assayed. Both titers reached a plateau at a level approximately 30% of the peak at around 3 months following the third injection and remained stable for the duration of this 7.5-month period. These animals were subsequently challenged with aerosolized anthrax spores (100-fold the LD_{50}), and all ten survived. At that time, post-challenge antibody titers increased in the surviving animals, suggesting that some spore germination did occur [37••]. 'Sterile immunity' is a time-limited immunological state since rabbits challenged 13 weeks post immunization displayed 'sterile immunity', but rabbits challenged after 7.5 months showed increases in titer. Results of these studies supported the further clinical development of a bivalent pDNA vaccine against anthrax.

Phase I clinical trial of a plasmid-based anthrax vaccine

The anthrax pDNA vaccine described above progressed in just 13 months from concept to the start of preclinical safety studies in support of IND filing. This rapid development was facilitated by the large body of research on *B anthracis* regarding the mechanism of toxicity, antigens that provide a protective immune response, *in vitro* assays for toxin-neutralizing antibody response, and animal models for critical *in vivo* challenge studies. However, even for less well-characterized targets than anthrax, pDNA vaccine technology can significantly shorten the time needed to select a vaccine candidate and begin preclinical studies.

A dose-escalation NIAID-supported trial has been initiated at two Vaccine and Treatment Evaluation Units (VTEUs) in 2004. In addition to safety, immunogenicity parameters including anti-PA and anti-LF antibody titers and *in vitro* Letx neutralization titers will be measured. Given that pDNA vaccines have induced cellular responses in humans [38], this clinical study represents an important measure of whether cationic lipid-formulated pDNA vaccines can also induce sufficient humoral responses in humans to advance pDNA into other antibody-mediated vaccine applications.

Conclusion

DNA vaccines have many properties that make them an attractive platform for biodefense applications: (i) speed of development, (ii) safety in humans, (iii) the ability to produce multi-agent and multivalent vaccines, (iv) uniformity of manufacturing and testing, and (v) the potential to produce vaccines with a balanced immune response. A pDNA vaccine for anthrax has been developed and has entered phase I clinical trial in a significantly shorter timeframe than traditional vaccine development. The full potential of DNA vaccines will be realized with the generation of a robust humoral immune response in humans. Cationic lipid pDNA formulations have shown great promise in preclinical animal models and are being evaluated in humans with a bivalent anthrax pDNA vaccine.

Acknowledgements

The authors would like to thank Drs David Kaslow, Peter Hobart, Carl Wheeler, Alan Dow, Tom Evans and Mr Alan Engbring for their valuable review of the manuscript.

References

•• of outstanding interest
• of special interest

1. Mock M, Fouet A: **Anthrax.** *Annu Rev Microbiol* (2001) **55**:647-671.
•• *This is a comprehensive review of anthrax disease, organism, toxins and pathology.*

2. Todar K: **Bacillus anthracis and anthrax.** In: *Online Textbook of Bacteriology.* University of Wisconsin-Madison, Madison, WI, USA (2001) (*http://www.textbookofbacteriology.net/Anthrax.html*).

3. Dixon TC, Meselson M, Guillemin J, Hanna PC: **Anthrax.** *N Engl J Med* (1999) **341**(11):815-826.

4. Inglesby TV, O'Toole T, Henderson DA, Bartlett JG, Ascher MS, Eitzen E, Friedlander AM, Gerberding J, Hauer J, Hughes J, McDade J *et al*: **Anthrax as a biological weapon, 2002: Updated recommendations for management.** *J Am Med Assoc* (2002) **287**(17):2236-2252.

5. Hoffmaster AR, Ravel J, Rasko DA, Chapman GD, Chute MD, Marston CK, De BK, Sacchi CT, Fitzgerald C, Mayer LW, Maiden MC *et al*: **Identification of anthrax toxin genes in a *Bacillus cereus* associated with an illness resembling inhalation anthrax.** *Proc Natl Acad Sci USA* (2004) **101**(22):8449-8454.

6. Mock M, Mignot T: **Anthrax toxins and the host: A story of intimacy.** *Cell Microbiol* (2003) 5(1):15-23.
• *This paper provides an updated review of anthrax toxin regulation and mechanism of action.*

7. Bradley KA, Mogridge J, Mourez M, Collier RJ, Young JA: **Identification of the cellular receptor for anthrax toxin.** *Nature* (2001) 414(6860):225-229.

8. Klimpel KR, Molloy SS, Thomas G, Leppla SH: **Anthrax toxin protective antigen is activated by a cell surface protease with the sequence specificity and catalytic properties of furin.** *Proc Natl Acad Sci USA* (1992) 89(21):10277-10281.

9. Singh Y, Chaudhary VK, Leppla SH: **A deleted variant of *Bacillus anthracis* protective antigen is non-toxic and blocks anthrax toxin action *in vivo*.** *J Biol Chem* (1989) 264(32):19103-19107.

10. Mogridge J, Cunningham K, Lacy DB, Mourez M, Collier RJ: **The lethal and edema factors of anthrax toxin bind only to oligomeric forms of the protective antigen.** *Proc Natl Acad Sci USA* (2002) 99(10):7045-7048.

11. Duesbery NS, Webb CP, Leppla SH, Gordon VM, Klimpel KR, Copeland TD, Ahn NG, Oskarsson MK, Fukasawa K, Paull KD, Vande Woude GF: **Proteolytic inactivation of MAP-kinase-kinase by anthrax lethal factor.** *Science* (1998) 280(5364):734-737.

12. Moayeri M, Haines D, Young HA, Leppla SH: ***Bacillus anthracis* lethal toxin induces TNF-α-independent hypoxia-mediated toxicity in mice.** *J Clin Invest* (2003) 112(5):670-682.

13. Tigerti WD: **Anthrax. William Smith Greenfield, M.D., F.R.C.P., Professor Superintendent, the Brown Animal Sanatory Institution (1878-81). Concerning the priority due to him for the production of the first vaccine against anthrax.** *J Hyg (Lond)* (1980) 85(3):415-420.

14. Friedlander AM, Welkos SL, Ivins BE: **Anthrax vaccines.** *Curr Top Microbiol Immunol* (2002) 271:33-60.
• *This paper reviews bacterial anthrax vaccine history and recent protein vaccine and adjuvant research.*

15. Brachman PS, Adimora AA, Berry SH, Bush T, Eickhoff TC, Ferrieri P, Gotschlich EC, Hilleman M, Kasper DL, Lockshin MD, Madigan D *et al*: *An Assessment of the CDC Anthrax Vaccine Safety and Efficacy Research Program.* The National Academies Press, Washington, DC, USA (2003) (*http://www.nap.edu/books/0309086264/html/*).

16. Turnbull PC, Leppla SH, Broster MG, Quinn CP, Melling J: **Antibodies to anthrax toxin in humans and guinea pigs and their relevance to protective immunity.** *Med Microbiol Immunol* (1988) 177(5):293-303.

17. Wiesen AR, Littell CT: **Relationship between prepregnancy anthrax vaccination and pregnancy and birth outcomes among US Army women.** *J Am Med Assoc* (2002) 287(12):1556-1560.

18. Joellenbeck L, Zwanziger LL, Durch JS, Strom BL: *The Anthrax Vaccine, Is It Safe? Does It Work?* The National Academies Press, Washington, DC, USA (2002) (*http://www.nap.edu/books/0309083095/html/*).

19. Jernigan DB, Raghunathan PL, Bell BP, Brechner R, Bresnitz EA, Butler JC, Cetron M, Cohen M, Doyle T, Fischer M, Greene C *et al*: **Investigation of bioterrorism-related anthrax, United States, 2001: Epidemiologic findings.** *Emerging Infect Dis* (2002) 8(10):1019-1028.

20. National Institute of Allergy and Infectious Diseases: **NIAID Biodefense Research.** *World Wide Web Site* (*http://www2.niaid.nih.gov/biodefense/*).

21. Office of Extramural Research at the National Institutes of Health: **Computer Retrieval of Information on Scientific Projects.** *World Wide Web Site* (*http://crisp.cit.nih.gov/*).

22. Ivins BE, Pitt ML, Fellows PF, Farchaus JW, Benner GE, Waag DM, Little SF, Anderson GW Jr, Gibbs PH, Friedlander AM: **Comparative efficacy of experimental anthrax vaccine candidates against inhalation anthrax in rhesus macaques.** *Vaccine* (1998) 16(11-12):1141-1148.

23. Filmore D: **Taking care of biosecurity business.** *Modern Drug Discovery* (2004) 7:36-42.

24. Donnelly JJ, Ulmer JB, Liu MA: **DNA vaccines.** *Dev Biol Stand* (1998) 95:43-53.

25. Gu ML, Leppla SH, Klinman DM: **Protection against anthrax toxin by vaccination with a DNA plasmid encoding anthrax protective antigen.** *Vaccine* (1999) 17(4):340-344.
•• *This is the first demonstration of a pDNA vaccine against anthrax PA being immunogenic and able to protect mice from an iv Letx toxin challenge.*

26. Williamson ED, Beedham RJ, Bennett AM, Perkins SD, Miller J, Baillie LW: **Presentation of protective antigen to the mouse immune system: immune sequelae.** *J Appl Microbiol* (1999) 87(2):315-317.

27. Boyle JS, Koniaras C, Lew AM: **Influence of cellular location of expressed antigen on the efficacy of DNA vaccination: Cytotoxic T lymphocyte and antibody responses are suboptimal when antigen is cytoplasmic after intramuscular DNA immunization.** *Int Immunol* (1997) 9(12):1897-1906.

28. Price BM, Liner AL, Park S, Leppla SH, Mateczun A, Galloway DR: **Protection against anthrax lethal toxin challenge by genetic immunization with a plasmid encoding the lethal factor protein.** *Infect Immun* (2001) 69(7):4509-4515.
•• *This paper reports that LF delivered by pDNA can protect mice from iv Letx toxin challenge and that LF, in addition to PA, is beneficial for a protective immune response.*

29. Galloway D, Liner A, Legutki J, Mateczun A, Barnewall R, Estep J: **Genetic immunization against anthrax.** *Vaccine* (2004) 22(13-14):1604-1608.

30. Goldenthal KL: **Prospective on the proposed rule.** *World Wide Web Site* (2004) (*www.fda.gov/cber/summaries/120600bio19KG.ppt*).
• *This is an FDA presentation of the new animal rule required for approval of vaccines that cannot ethically be tested on humans.*

31. Hedstrom RC, Doolan DL, Wang R, Gardner MJ, Kumar A, Sedegah M, Gramzinski RA, Sacci JB Jr, Charoenvit Y, Weiss WR, Margalith M *et al*: **The development of a multivalent DNA vaccine for malaria.** *Springer Semin Immunopathol* (1997) 19(2):147-159.
• *This study demonstrates that application of the multivalent vaccine approach is made possible with pDNA vaccines.*

32. Riemenschneider J, Garrison A, Geisbert J, Jahrling P, Hevey M, Negley D, Schmaljohn A, Lee J, Hart MK, Vanderzanden L, Custer D *et al*: **Comparison of individual and combination DNA vaccines for *B. anthracis*, Ebola virus, Marburg virus and Venezuelan equine encephalitis virus.** *Vaccine* (2003) 21(25-26):4071-4080.
• *This paper presents a bioterrorism vaccine application of the multivalent pDNA vaccine approach.*

33. Payne LG, Fuller DH, Haynes JR: **Particle-mediated DNA vaccination of mice, monkeys and men: Looking beyond the dogma.** *Curr Opin Mol Ther* (2002) 4(5):459-466.

34. Roy MJ, Wu MS, Barr LJ, Fuller JT, Tussey LG, Speller S, Culp J, Burkholder JK, Swain WF, Dixon RM, Widera G *et al*: **Induction of antigen-specific CD8+ T cells, T helper cells, and protective levels of antibody in humans by particle-mediated administration of a hepatitis B virus DNA vaccine.** *Vaccine* (2000) 19(7-8):764-778.

35. Tucker SN, Lin K, Stevens S, Scollay R, Bennett MJ, Olson DC: **Systemic and mucosal antibody responses following retroductal gene transfer to the salivary gland.** *Mol Ther* (2003) 8(3):392-399.

36. Fischer L, Minke J, Dufay N, Baudu P, Audonnet JC: **Rabies DNA vaccine in the horse: Strategies to improve serological responses.** *Vaccine* (2003) 21(31):4593-4596.
• *This study demonstrates the pDNA vaccine humoral immune response-enhancing activity of cationic lipids in a large animal model.*

37. Hermanson G, Whitlow V, Parker S, Tonsky K, Rusalov D, Ferrari M, Lalor P, Komai M, Mere R, Bell M, Brenneman K *et al*: **A cationic lipid-formulated plasmid DNA vaccine confers sustained antibody-mediated protection against aerosolized anthrax spores.** *Proc Natl Acad Sci USA* (2004) 101(37):13601-13606.
•• *This paper presents the development and testing of a bivalent pDNA vaccine against anthrax in a relevant animal model. Pre- and post-challenge immune response and survival are determined at peak titer and extended timepoints.*

38. Wang R, Doolan DL, Le TP, Hedstrom RC, Coonan KM, Charoenvit Y, Jones TR, Hobart P, Margalith M, Ng J, Weiss WR *et al*: **Induction of antigen-specific cytotoxic T lymphocytes in humans by a malaria DNA vaccine.** *Science* (1998) 282(5388):476-480.

39. Hartikka J, Bozoukova V, Ferrari M, Sukhu L, Enas J, Sawdey M, Wloch MK, Tonsky K, Norman J, Manthorpe M, Wheeler CJ: **Vaxfectin enhances the humoral immune response to plasmid DNA-encoded antigens.** *Vaccine* (2001) 19(15-16):1911-1923.
• *This paper describes the humoral immune response enhancement of cationic lipids for pDNA vaccines.*

40. Reyes L, Hartikka J, Bozoukova V, Sukhu L, Nishioka W, Singh G, Ferrari M, Enas J, Wheeler CJ, Manthorpe M, Wloch MK *et al.*: **Vaxfectin enhances antigen specific antibody titers and maintains Th1 type immune responses to plasmid DNA immunization.** *Vaccine* (2001) 19(27):3778-3786.
• *This study provides further characterization of the immune response enhancement of cationic lipid formulated pDNA vaccines.*

41. Nukuzuma C, Ajiro N, Wheeler CJ, Konishi E: **Enhancing effect of vaxfectin on the ability of a Japanese encephalitis DNA vaccine to induce neutralizing antibody in mice.** *Viral Immunol* (2003) 16(2):183-189.

42. Locher CP, Witt SA, Ashlock BM, Levy JA: **Evaluation of genetic immunization adjuvants to improve the effectiveness of a human immunodeficiency virus type 2 (HIV-2) envelope DNA vaccine.** *DNA Cell Biol* (2004) 23(2):107-110.

43. Locher CP, Witt SA, Ashlock BM, Polacino P, Hu SL, Shiboski S, Schmidt AM, Agy MB, Anderson DM, Staprans SI, zur Megede J, Levy JA: **Human immunodeficiency virus type 2 DNA vaccine provides partial protection from acute baboon infection.** *Vaccine* (2004) 22(17-18):2261-2272.

44. Sankar V, Baccaglini L, Sawdey M, Wheeler CJ, Pillemer SR, Baum BJ, Atkinson JC: **Salivary gland delivery of pDNA-cationic lipoplexes elicits systemic immune responses.** *Oral Dis* (2002) 8(6):275-281.

45. D'Souza S, Rosseels V, Denis O, Tanghe A, De Smet N, Jurion F, Palfliet K, Castiglioni N, Vanonckelen A, Wheeler C, Huygen K *et al.*: **Improved tuberculosis DNA vaccines by formulation in cationic lipids.** *Infect Immun* (2002) 70(7):3681-3688.

46. Fischer L, Tronel JP, Minke J, Barzu S, Baudu P, Audonnet JC: **Vaccination of puppies with a lipid-formulated plasmid vaccine protects against a severe canine distemper virus challenge.** *Vaccine* (2003) 21(11-12):1099-1102.

47. Jiao X, Wang RY, Feng Z, Alter HJ, Shih JW: **Modulation of cellular immune response against hepatitis C virus nonstructural protein 3 by cationic liposome encapsulated DNA immunization.** *Hepatology* (2003) 37(2):452-460.

48. Ishii N, Fukushima J, Kaneko T, Okada E, Tani K, Tanaka SI, Hamajima K, Xin KQ, Kawamoto S, Koff W, Nishioka K *et al.*: **Cationic liposomes are a strong adjuvant for a DNA vaccine of human immunodeficiency virus type 1.** *AIDS Res Hum Retroviruses* (1997) 13(16):1421-1428.

49. Okada E, Sasaki S, Ishii N, Aoki I, Yasuda T, Nishioka K, Fukushima J, Miyazaki J, Wahren B, Okuda K: **Intranasal immunization of a DNA vaccine with IL-12- and granulocyte-macrophage colony-stimulating factor (GM-CSF)-expressing plasmids in liposomes induces strong mucosal and cell-mediated immune responses against HIV-1 antigens.** *J Immunol* (1997) 159(7):3638-3647.

50. Toda S, Ishii N, Okada E, Kusakabe KI, Arai H, Hamajima K, Goral I, Nishioka K, Okuda K: **HIV-1-specific cell-mediated immune responses induced by DNA vaccination were enhanced by mannan-coated liposomes and inhibited by anti-interferon-γ antibody.** *Immunology* (1997) 92(1):111-117.

51. Yokoyama M, Zhang J, Whitton JL: **DNA immunization: Effects of vehicle and route of administration on the induction of protective antiviral immunity.** *FEMS Immunol Med Microbiol* (1998) 14(4):221-230.

52. Gramzinski RA, Millan CL, Obaldia N, Hoffman SL, Davis HL: **Immune response to a hepatitis B DNA vaccine in *Aotus* monkeys: A comparison of vaccine formulation, route, and method of administration.** *Mol Med* (1998) 4(2):109-118.

53. Perrie Y, Frederik PM, Gregoriadis G: **Liposome-mediated DNA vaccination: The effect of vesicle composition.** *Vaccine* (2001) 19(23-24):3301-3310.

54. Wong JP, Zabielski MA, Schmaltz FL, Brownlee GG, Bussey LA, Marshall K, Borralho T, Nagata LP: **DNA vaccination against respiratory influenza virus infection.** *Vaccine* (2001) 19(17-19):2461-2467.

55. Sha Z, Vincent MJ, Compans RW: **Enhancement of mucosal immune responses to the influenza virus HA protein by alternative approaches to DNA immunization.** *Immunobiology* (1999) 200(1):21-30.